THE ENIGMATIC PROPERTIES OF FLUORESCENT BANDING IN MASSIVE CORALS OF THE SPECIES *PORITES LUTEA* FROM PHUKET, THAILAND

BY

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My gratitude is also extended to those in the Newcastle Research Group, and in particular to Dr. Andrew Bishop who refers to the yellow/orange fluorescent patches as Mr Blobby 1, 2, 3 etc. Thanks for all the time you spent doing me favours. Chapter 5 is about twice the size as a consequence.

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ABSTRACT

Bright bands in massive corals of the species *Porites lutea* have been associated with periods of increased rainfall. However, stain experiments involving *Porites lutea* from Ko Phuket have shown that bright bands are deposited during the dry season. Thus, one of the main objectives of this thesis was to understand more fully the processes that are involved in the production of fluorescent bands. The second major objective was to build a device that could reliably record fluorescent emissions directly from solid coral. It was hoped that the fluorescent emissions recorded in this way could then be compared with environmental factors, and in particular rainfall.

In order to achieve the first objective, solid state coral fluorescence was examined to determine (1) what effects porosity (macro and micro) have on coral fluorescence, and (2) the distribution of fluorophores in the skeleton. By studying the fluorescence of solutions in which the $CaCO_3$ had been removed by acid dissolution, it was possible to determine the effects of both fluorophore concentration and the metal ions, iron and manganese, on coral fluorescence. Environmental samples (soil, sediment, seawater and polyp tissue) were also examined in an attempt to identify the source(s) of coral fluorophores. Although Boto and Isdale (1985) have suggested that terrestrial humic acid is responsible for bright band fluorescence in *Porites lutea*, the same, or very similar, fluorophores were found in both bright and dull bands. Two main types of fluorophore were identified, a 330-340 nm excitation peak group and a 390 nm excitation peak group. Work has shown that changes in the absolute concentration of these fluorophores (i.e. their relative concentrations remain constant) appears to be the main control on fluorescent banding. Increases in fluorophores concentration can result in both a change in the colour of coral fluorescence as well as a change in intensity. When solid coral was viewed using a fluorescence microscope, two main types of fluorescence were seen, yellow/orange fluorescent patches and a blue background fluorescence. The yellow/orange fluorescent patches were randomly distributed, ranged in size from 8-35 μm and appeared to be due to black, possibly organic, inclusions (sub-micron to 3 μm) . Although the distribution of these patches was not mapped, observations suggested that they were more numerous in bright bands. This thesis suggests that differences in the ratio of yellow/orange fluorescent patches to background fluorescence controls the **colour** of coral fluorescence. As fluorescent emissions in the solid state are dominated by short wavelength emissions, the background region of the coral (which is also dominated by short wavelength emissions) is thought to exert the main control on fluorescent **intensity**. Although terrestrial surface soil contained the most concentrated source of coral-like fluorophores, it was not possible to confirm the origin of coral fluorescence as coral-like fluorophores were found in all the environmental samples.

In order to achieve the second objective, incident radiation from a mercury-argon lamp, chopped to a specific wavelength using a bandpass filter, was used to excite the coral surface. Emissions were recorded at specific wavelength by a photomultiplier. Using a stage assembly attached to a stepper motor, coral skeleton was passed in front of the u/v beam at 1.25 mm/minute along a fixed path. Results demonstrated that bright bands were, in general, more intense than their neighbouring dull bands. However, trends towards higher or lower fluorescent intensity with increasing distance from the living coral surface questioned the validity of the data. These trends were not thought to be due to instrumental drift as the errors associated with profiles taken first one way and then the other, were low (less than 5%). Suggestions for the trends include: microbial activity, photo-oxidation, and ageing of the fluorophores. Even when the trends were removed by fitting an exponential curve, poor correlation's were produced between coral profiles once the seasonality had been removed, even for corals from the same reef. This was thought to be due to (1) the heterogeneous distribution of fluorophores in the skeleton, (2) porosity/corallite orientation, (3) the small amount of data generated. Thus, at present, the data produced by the fluorescence measuring device was not of a sufficiently high standard for a meaningful comparison with environmental parameters, such as rainfall, to be made.

The discovery of fluorescent banding in *Porites spp.* from Oman, which receives no terrestrial input, has questioned previous assumptions that fluorescent banding is due to seasonal variations in the supply of terrestrial fluorophores into the marine environment. For the Phuket corals, the most plausible explanation that could account for bright band deposition in the dry season (either with or without the presence of terrestrial fluorophores) relates to seasonal variations in calcification rate. Results show that dull band calcification rate is greater than bright band calcification rate in the Thai corals. Thus, assuming that the rate of fluorophore incorporation into the skeleton is constant throughout the year, the ratio of coral fluorophores to carbonate is likely to be higher in bright bands which could be why they are bright.

ARCHIVE

Except where stated, all corals in this list are *Porites lutea*.

Sample and sample site:	Collected by	Date of collection
Tin Smelter Reef:		
TS-1-93	Theodorou	August 1993
TS-3-93	Theodorou	August 1993
TS-3-89	Scoffin and Tudhope	August 1989
TS-8-88	Scoffin and Tudhope	January 1988
TS-10-88	Scoffin and Tudhope	January 1988
TS-1-87	Scoffin and Tudhope	August 1987
TS-4-87	Scoffin and Tudhope	August 1987
TS-5-87	Scoffin and Tudhope	August 1987
TS-9-87	Scoffin and Tudhope	August 1987
TS-25-87	Scoffin and Tudhope	August 1987
Porites Bay:		
PB-5-93	Theodorou	August 1993
PB-1-90	Scoffin and Tudhope	January 1990
PB-2-90	Scoffin and Tudhope	January 1990
PB-3-90	Scoffin and Tudhope	January 1990
PB-6-90	Scoffin and Tudhope	January 1990
PB-2-87	Scoffin and Tudhope	August 1987
PB-4-87	Scoffin and Tudhope	August 1987
PB-3-87	Scoffin and Tudhope	August 1987
PB-8-87	Scoffin and Tudhope	August 1987
Other species		
Goniastrea retiformis	Theodorou	August 1993
Platygyra daedalea	Theodorou	August 1993

Sample and sample site:	Collected by	Date of collection
Shark Bay:		
SB-1-92	Theodorou	July 1992
SB-1A-88	Scoffin and Tudhope	January 1988
SB-6-88	Scoffin and Tudhope	January 1988
SB-10-88	Scoffin and Tudhope	January 1988
Aquarium Reef:		
AQ-7-92	Theodorou	July 1993
AQ-1-92 Control	Theodorou	July 1992
AQ-1-92 Mn stain	Theodorou	July 1992
AQ-1-92 Fe stain	Theodorou	July 1992
AQ-1-88	Scoffin and Tudhope	January 1988
AQ-4-87	Scoffin and Tudhope	August 1987
AQ-15-87	Scoffin and Tudhope	August 1987
AQ-25-87	Scoffin and Tudhope	August 1987
AQ-35-87	Scoffin and Tudhope	August 1987
Core 9/1/87	Scoffin and Tudhope	August 1987
Similan Islands:		
S-2-90	Scoffin and Tudhope	January 1990
S-5-90	Scoffin and Tudhope	January 1990
S-6-90	Scoffin and Tudhope	January 1990

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CHAPTER 1. INTRODUCTION

CHAPTER 1. INTRODUCTION

In this thesis I shall discuss the nature, origin and significance of fluorescence in coral skeletons of the species *Porites lutea* from Ko Phuket, South Thailand.

1.1 CORALS AS ENVIRONMENTAL RECORDERS:

Hermatypic (reef building) corals record in the composition and structure of their skeleton information about many of the physical and chemical conditions that prevailed during their growth. Because individual massive coral colonies can survive for several centuries, have linear extension rates between 1-3 cm/year, live in a wide range of environments, are relatively resistant to environmental change, and have distinctive annual skeletal banding, they can be used to monitor spatial and temporal variations in environmental conditions. To date, they have provided information on rainfall, seawater temperature, solar radiation and oceanic upwelling.

1.2 PREVIOUS WORK:

Sclerochronology is the name given to the study of seasonal banding within coral skeletons (analogous to dendrochronology in trees) and provides a useful tool for accurately dating past changes in the marine environment (both local and tropical). Seasonal banding patterns come in a variety of forms and can be broadly divided into chemical (see Scoffin 1989 for full review), density (discussed in Chapter 2, section 2.2.1, page 25) and fluorescent banding.

1.2.1 Fluorescent banding: Fluorescent banding was first identified by Isdale (1984) when massive corals of the species *Porites lutea* from the Great Barrier Reef were excited with longwave u/v light. Since then, fluorescent banding has been found in corals of the same genus in Florida Bay (Smith *et al* 1989), Papua New Guinea and Indonesia (Scoffin *et al* 1989), Sinai (Klein *et al* 1990), South Thailand (Scoffin *et al* 1992) and Oman (Tudhope *et al* unpublished). The terms green/yellow and blue, bright and dull, bands and non bands, have all been loosely used to described fluorescent bands. However, distinguishing between brightness and colour is not always a simple matter as the brighter a colour, the lighter it actually appears.

Although it would appear that fluorescent banding is associated with variations in intensity, it is far from clear whether it is also associated with variations in colour. Thus, the terms bright and dull are used to describe fluorescent bands in this study (see Figure 1.1).

Figure 1.1 Fluorescent banding in *Porites lutea* from Ko Phuket when excited with longwave u/v light. (BB) = bright band and (DB) = dull band. This is a reef front coral which was collected from Tin Smelter Bay in August 1987 (TS-1-87). All photographs presented in this thesis were taken under u/v light (both black and white and colour), a u/v cut off was used to prevent scattered/reflected light from the source (i.e. u/v lamp) reaching the camera.

Fluorescent banding is a feature almost unique to massive corals. Although some branching corals do have fluorescent bands, they are generally poorly developed. The massive coral *Porites lutea* is the most widely used coral in fluorescent banding studies because its bands are visibly the best, a feature which may be due to its fine porous structure. Perhaps the most significant discovery with respect to fluorescent banding was made by Scoffin *et al* (1992). Using staining techniques, they discovered that one bright and one dull band (known as a fluorescent couplet) are, in general, deposited approximately every 12 months. Thus, it would appear that fluorescent banding is an annual phenomenon and as such may be of some environmental significance. The ability to use fluorescent banding in corals as a means of hindcasting environmental events depends on the coral's ability to reliably record environmental information. Scoffin *et al* (1989) reported that while corals from a particular reef have broadly similar fluorescent banding patterns (see Figure 1.2), it becomes increasingly difficult to correlate with confidence the banding patterns from coral reefs separated by more than 25 km as these reefs may be subject

to differing local environmental conditions. This potentially limits the use of fluorescent banding as an environmental indicator of local conditions.

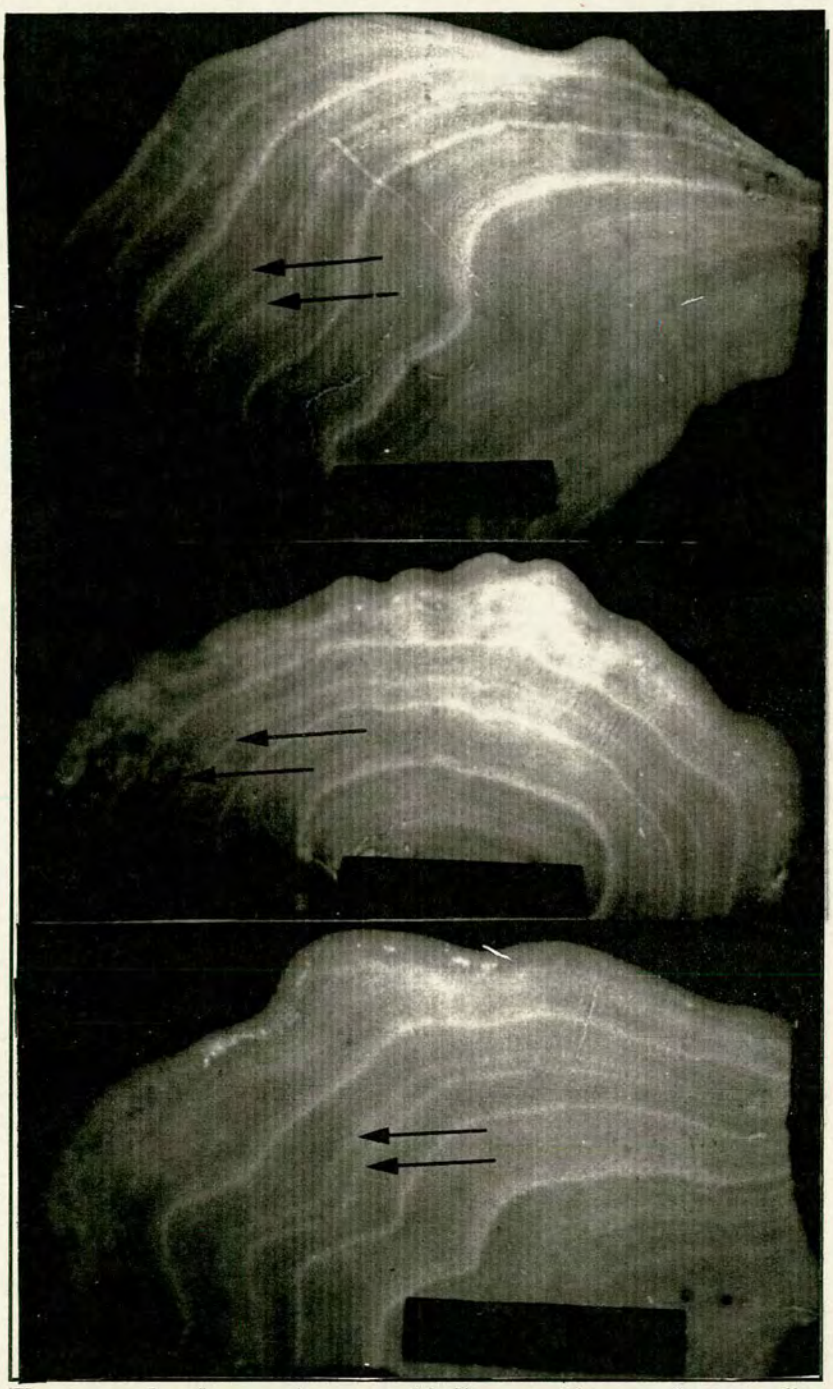


Figure 1.2 Fluorescent bands as environmental indicators. These corals were collected from three different corals from the Similan Islands in January 1990 by Tudhope and Scoffin (from top to bottom, S-2-90, S-5-90 and S-6-90). It can be seen that the fluorescent banding is similar in all three corals, with bright bands appearing with approximately the same frequency and intensity. For example, the last major bright band before the living coral surface is preceded by two faint bright bands which have been indicated in all three cases. This therefore implies that these corals are recording the same external signal. The scale bar is 5 cm long.

However, before fluorescent banding can be used as an environmental indicator, the reason why two types of fluorescent band are deposited annually has to be determined.

1.2.2 The causes of fluorescent banding: Isdale (1984) suggested that the close correlation between bright band deposition in inshore corals from the Great Barrier Reef and outflow from the Burdekin river suggested that bright bands were caused by the incorporation of terrestrially derived components. Boto and Isdale (1985) measured the fluorescent spectra of solid coral cores using a 150- W mercury arc excitation beam passed through a 360 nm bandpass filter. Quartz optical fibres were used to direct the excitation beam and collect fluorescent emissions which were passed into the monochromator and detector system of a Varian AA6 atomic absorption spectrophotometer. They found that emission spectra were very broad with a maxima at 440-460 nm, and suggested that this was indicative of humic and fulvic acids. Humic and fulvic acids form part of a larger group of compounds which are known as humic substances. The term "humic substance" describes a general category of compounds which have been defined as naturally occurring, biogenic, heterogeneous organic substances (Aiken *et al* 1985). Although a considerable amount of research has been carried out on humic substances, very little is known about them. As it was not the aim of this thesis to understand more fully the processes involved in the formation of humic substances or to characterise their chemical structure, it was not considered necessary to discuss such matters in the main body of the text (see Appendix A1.1 for full discussion). This thesis makes no effort to differentiate between humic and fulvic acids and uses the term humic acid exclusively. This is because work by Susic *et al* (1991) failed to detect a significant difference in the chemical properties of humic and fulvic acids.

In order to test the hypothesis that coral fluorescence is due to the presence of humic acids in the crystal lattice, Boto and Isdale (1985) performed a number of simple experiments all of which involved the extraction of the humics from the lattice.

(a) Dissolution of coral samples in dilute HCl gave solutions which showed broad fluorescence excitation and emission spectra typical of humic acids.

(b) Solutions with similar characteristics to those in (a) were obtained by hot alkali (0.1M NaOH) extraction of the crushed coral skeleton, thus implying the existence of organic acid functional groups.

(c) The addition of excess Fe^{3+} to solutions obtained by acid dissolution or alkaline extraction of the coral shifted the emission spectrum to longer wavelengths and reduced fluorescent intensity by ~30%. In other words, coral fluorescence is quenched by iron, a characteristic which is also typical of humic acids.

(d) Coral solutions obtained by acid dissolution were passed over Amberlite XAD-2 resin which has a strong affinity for humic acids. It was found that 90% of the fluorescent components were absorbed on to this resin and could be eluted with NH_4OH (pH 11).

Thus, it appeared that humic acids were responsible for coral fluorescence. Comparison of bright and dull band emission spectra showed that maximum differences occurred in the 480-530 nm region (see Figure 1.3).

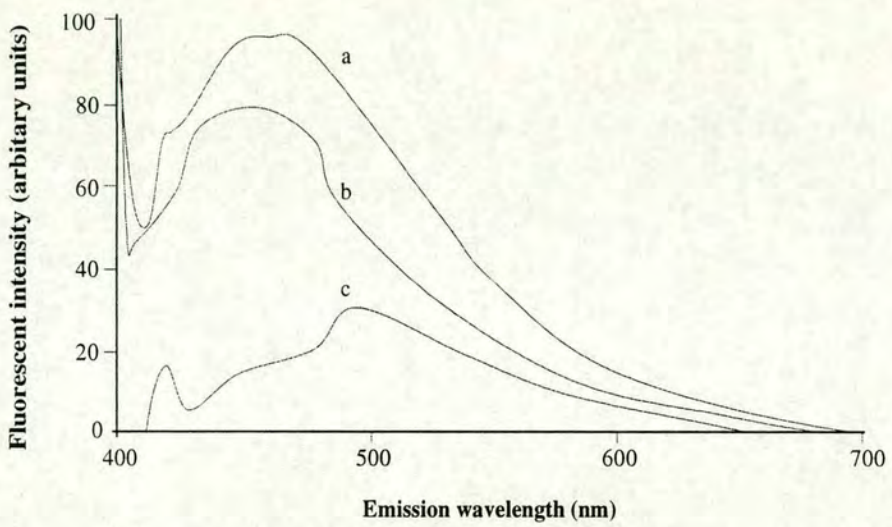


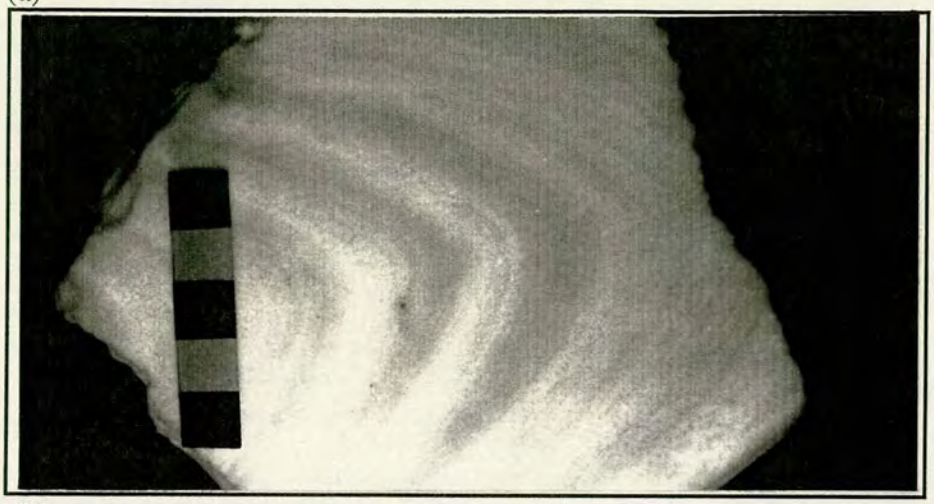
Figure 1.3 Emission spectra from solid state bright and dull bands. Data taken from Boto and Isdale (1985) Key: a, bright band and b, dull band regions of an inshore *Porites spp.* coral skeleton. c, direct subtraction of b from a.

Boto and Isdale (1985) suggest that these differences, although small, combined with the human eye response, result in the yellow/green appearance of the bright fluorescent bands. They went on to suggest that this difference was due to two types of humic acid, marine and terrestrial[1]. They suggest that while all parts of the skeleton contain marine humic acids which impart a blue background fluorescence, the yellow/green fluorescent bands result from enhanced concentrations of low

[1]Although it was originally believed that all humic acids were terrestrial in origin (Degens *et al* 1964), plenty of evidence now exists which demonstrates the existence of a marine source. Further details are given in Appendix A1.1).

relative molecular mass humic acids (known as fulvic acids) from a terrestrial source. They believe that terrestrial humic acids are washed into the marine environment during the wet season and incorporated into the coral skeleton along with marine humic acids. This explanation was then used to account for a similar relationship between bright band deposition and periods of high rainfall in corals of the same species from Papua New Guinea and Indonesia (Scoffin *et al* 1989). Evidence to support the hypothesis that bright bands result from increased concentrations of terrestrially-derived humic acids was provided by Susic *et al* (1991) who worked on *Porites lutea* from the Great Barrier Reef. They found that bright bands (which are deposited in the wet season in this area) contained a higher concentration of humic acids than dull bands (65.4 ppm as opposed to 46.2 ppm respectively) and that this correlated with an increase in the concentration of humic acids in seawater at this time. The fact that the concentration of humic acids in river water was between 2 and 10 times greater than in seawater and that even higher concentrations were found in soils, suggested that the rise in seawater humic acid concentration seen during the wet season was due to the presence of terrestrially-derived humic acids. Further evidence to support this hypothesis came from the discovery that the concentration of humic acid in coral skeletons decreased with increasing distance from the shore; a feature that was also found in seawater samples (Susic *et al* 1991). These results were consistent with visual observations which suggest that the intensity, size, and possibly even the number of bright bands decrease with increasing distance from the shore (Isdale 1984; and Scoffin *et al* 1989), a feature that is also seen in the Thai Study area (see Figure 1.4).

(a)



(b)

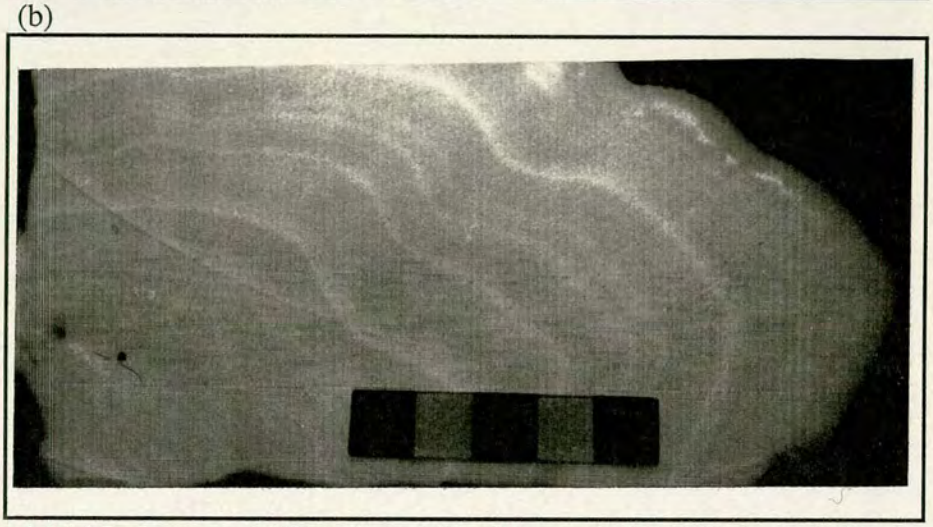


Figure 1.4 (a) Inshore coral (PB-1-90) and (b) offshore coral (S-6-90) from the Thai study area photographed under u/v light. The scale bar is 5 cm long.

Although it was originally thought that humic acids were entirely responsible for coral fluorescence, work by Susic *et al* (1991) on corals from the GBR has shown that in offshore corals, humic acids account for approximately 68% of the total fluorescence, while they account for 74% of the fluorescence in inshore bright bands and 70% in inshore dull bands. This therefore suggests that while humic acids are the dominant coral fluorophores[2], they are not the only fluorophores found in coral skeletons. Susic *et al* (1991) suggest that the remaining 25-30% is due to the presence of other fluorescent molecules such as non-humic metabolites from the coral itself. Dunlap and Susic (1985) have suggested that pteridines and flavins are the most likely fluorescent metabolites to be incorporated into coral skeletons as they

[2] A fluorophore is a molecule, or part of a molecule that emits fluorescence when excited by radiation, usually in the u/v or visible part of the spectrum.

are the most abundant terminal catabolic products excreted by marine organisms. This seems even more plausible given that Gentein (1981) has reported the occurrence of high levels of pteridines in coral reef waters and Foutaine *et al* (1981) have demonstrated that scleractinian corals release riboflavin.

Having discovered a link between rainfall and fluorescence, Boto and Isdale (1985) suggested that the size and intensity of bright bands related to the length of the wet season and how much it rained during that period. The longer and wetter the wet season, the thicker and brighter the bright band. Thus, it appeared that fluorescent banding could be used as a tool for hindcasting rainfall. Examples where fluorescent banding has been used in this way are as follows:

(a) Klein *et al* (1990) suggested that the presence of fluorescent banding in late Quaternary *Porites* from the Sinai desert and their absence in modern *Porites* indicated a wetter climate in the past in this region.

(b) Kotwicki and Isdale (1991) suggested that fluorescent banding could be used to hindcast El Niño events. As the El Niño Southern Oscillation, which can occur every 2-10 years (see Philander 1983 for a review), is though to be responsible for severe droughts in southern Africa, Australia, Indonesia and India, and floods in Ecuador, Northern Peru and the Gulf coast, an ability to predict when and how severe an event will be is of great importance.

To summarise, the deposition of bright fluorescent bands in coral skeletons of the species *Porites lutea* from a number of reefs around the world has been linked to the incorporation of terrestrially derived humic acids that are washed into the marine environment during the wet season.

1.3 THE RATIONALE BEHIND THE THESIS AND ITS OBJECTIVES:

Although previous work has strongly suggested that bright band fluorescence in *Porites lutea* is related to periods of increased rainfall, staining experiments performed by Tudhope and Scoffin in 1987, and by myself in 1992 suggest that bright bands in *Porites lutea* from Ko Phuket are deposited during the dry season (November to April) and dull bands are deposited during the wet season (April to November) (see Figure 1.5). The climate of the region is discussed in more detail in

section 1.4.1, page 13. In Figure 1.5a and b the coral skeleton was stained with alizarin by Tudhope and Scoffin in November 1986 and collected in August 1987. In each case it would appear that November marks the end of dull band deposition and the beginning of bright band deposition. The coral skeleton in Figure 1.5c was stained with $FeCl_2$ by myself in 1992. It can be seen that the iron band was deposited in a dull band. The closeness of the iron band to the bright/dull band boundary suggests that dull band deposition began around the beginning of July.

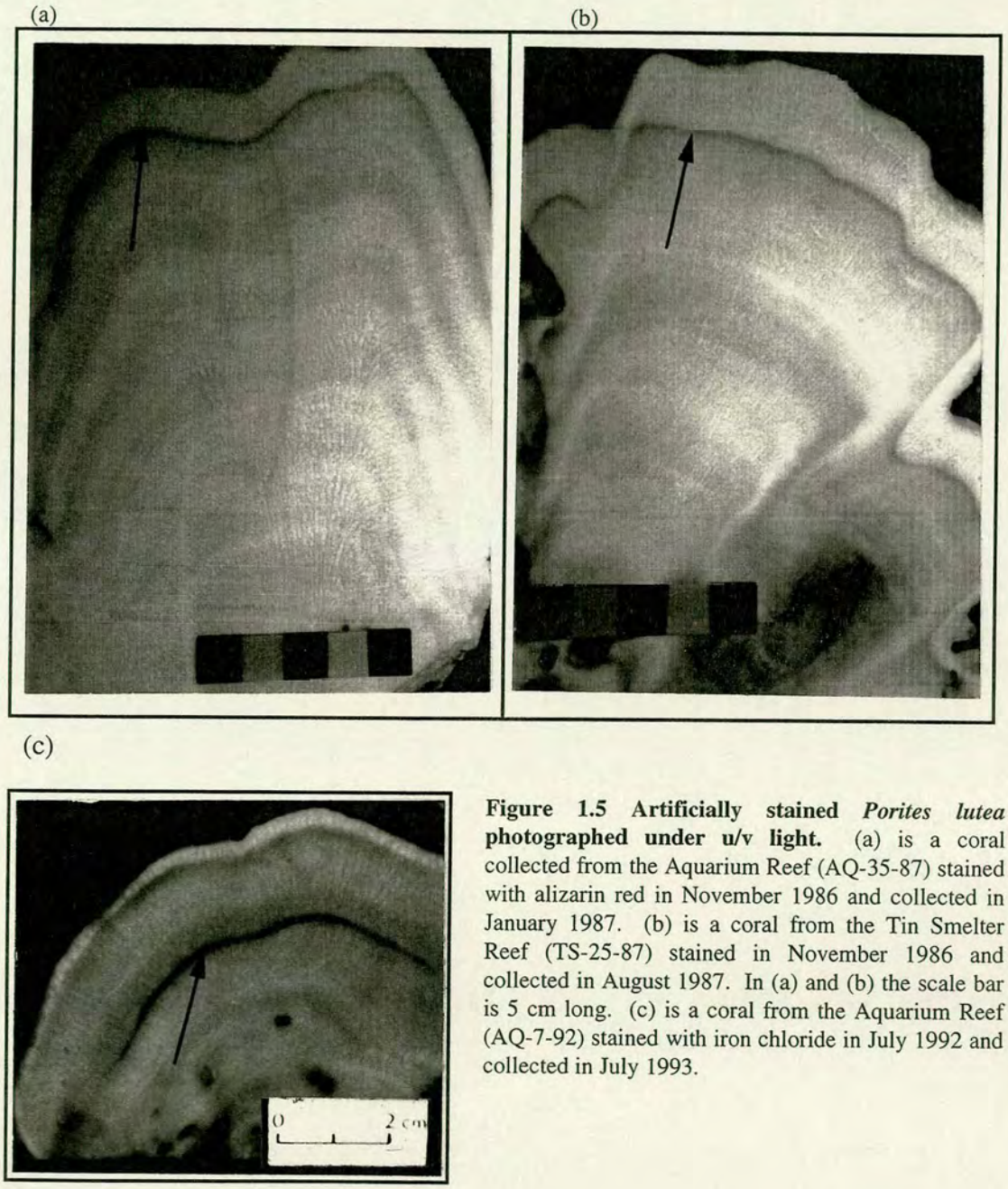


Figure 1.5 Artificially stained *Porites lutea* photographed under u/v light. (a) is a coral collected from the Aquarium Reef (AQ-35-87) stained with alizarin red in November 1986 and collected in January 1987. (b) is a coral from the Tin Smelter Reef (TS-25-87) stained in November 1986 and collected in August 1987. In (a) and (b) the scale bar is 5 cm long. (c) is a coral from the Aquarium Reef (AQ-7-92) stained with iron chloride in July 1992 and collected in July 1993.

Thus, these observations strongly suggest that dull bands are deposited in the wet season while bright bands are deposited in the dry season. In other words, the opposite relationship between fluorescent banding and rainfall is seen in the Thai corals. Therefore, if a relationship exists between coral fluorescence and rainfall in *Porites lutea* from Ko Phuket, it is not a straightforward one. In addition, fluorescent banding has also been detected in *Porites spp.* in areas that are free from the effects of terrestrial input such as in Oman (see Figure 1.6). This therefore questions the supposition that terrestrial humic acids are an essential prerequisite for coral fluorescence.

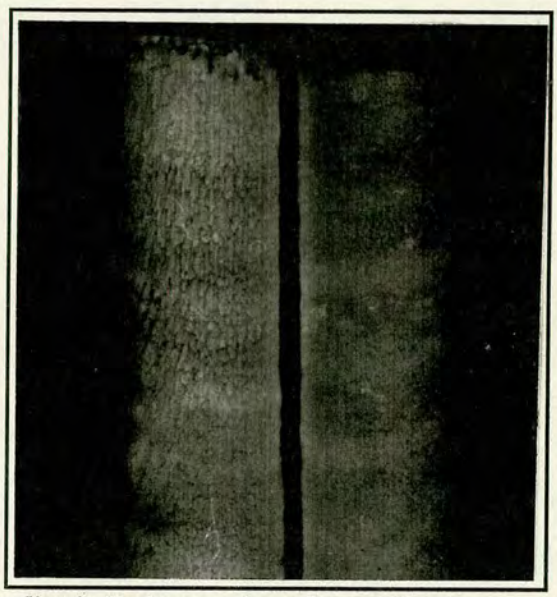


Figure 1.6 Fluorescent banding in *Porites spp.* from Oman. Coral cores approximately 10 cm long supplied by Tudhope.

Thus, the main aim of this thesis is to understand more fully the processes that are involved in the formation of fluorescent bands to enable us to answer the questions: (1) is fluorescent banding due to the presence of terrestrially derived humic acids? and (2) why are bright bands deposited in the dry season in the Thai area? The second major objective of this thesis was to build a device that could reliably record solid state fluorescence. It was hoped that the fluorescent profiles produced in this way could be compared with a variety of environmental records for which reliable data exists in the Thai area (e.g. rainfall, sunshine and productivity).

The rest of this chapter provides a brief description of the study area, lists the sample sites, and describes how corals were selected and treated before analysis.

1.4 DESCRIPTION OF THE STUDY AREA:

Ko Phuket is an Island approximately 40 km long on the south west coast of Thailand in the Andaman Sea at latitude 8°00'N and longitude 98°20'E (see Figure 1.7).

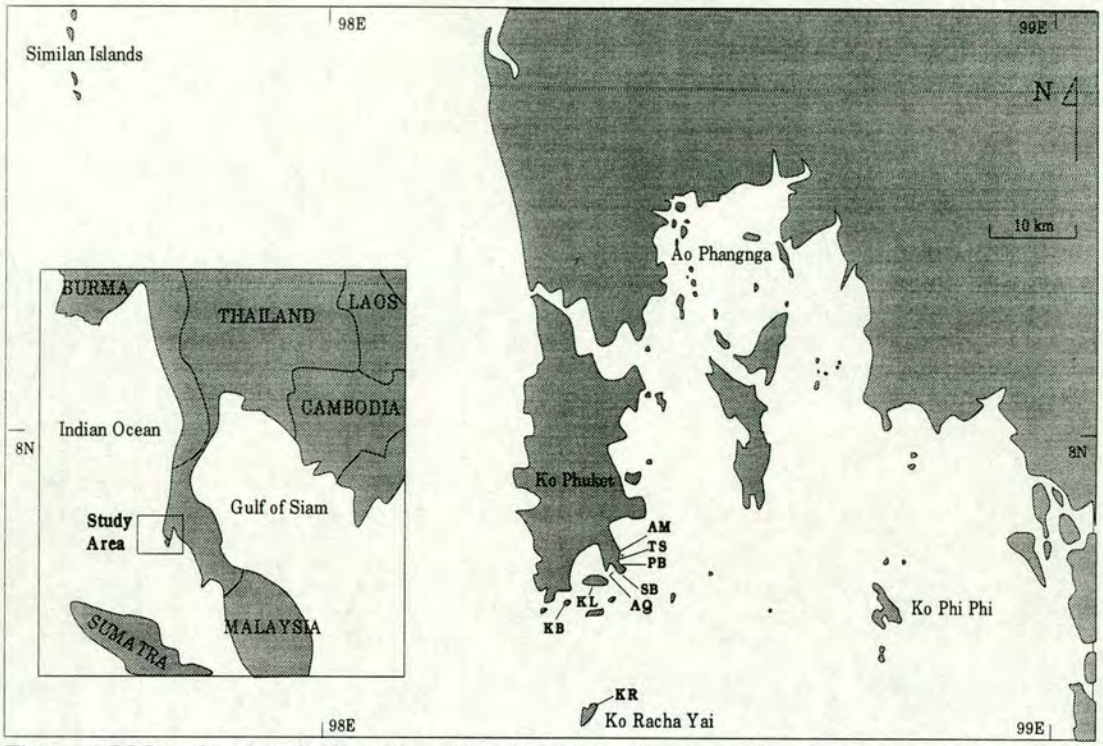


Figure 1.7 Map showing the location of the study area. Reef sampling sites are also indicated: AM = Ao Man, AQ = Aquarium reef, KB = Ko Bon, KL = Ko Lon, KR = Ko Racha, PB = Porites Bay, SB = Shark Bay, and TS = Tin Smelter Bay.

Tudhope and Scoffin (1994) have suggested that reef development in this region is substantially different to models of Holocene reef development in the better studied open-shelf and oceanic settings. They determined that the reefs in this area initiated growth on rocky coastal prominences after the present sea level was reached about 6,000 years ago and that through time they have prograded out over muddy sediments with living corals at the reef front and fossil corals becoming progressively older (dated using ^{14}C carbon isotope) in a shoreward direction (see Figure 1.8).

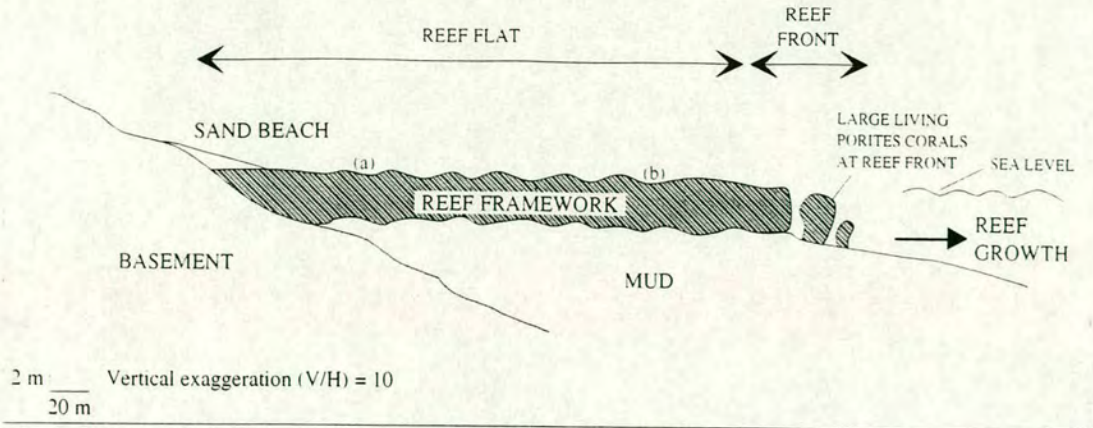


Figure 1.8 Cartoon of a section across the reef in Tang Khen Bay. The reef forms only a thin veneer overlaying muddy sediments. Tudhope and Scoffin (1994) suggested that the reefs in the Ko Phuket study area have prograded out from the shore over the past 6,000 years. Reef flat zone: (a) *Goniastrea astrea*, (b) *Goniastrea retiformis*.

An advantage of this type of reef setting is that fossil corals up to 6,000 years old are easily accessible. This is in contrast to most reefs which tend to grow upwards and outwards on top of older pre-existing corals.

1.4.1 Local climate: The study area has a monsoonal climate with a dry north-east (NE) monsoon from November to April and a wet south-west monsoon (SW) from April to November see (Figure 1.9).

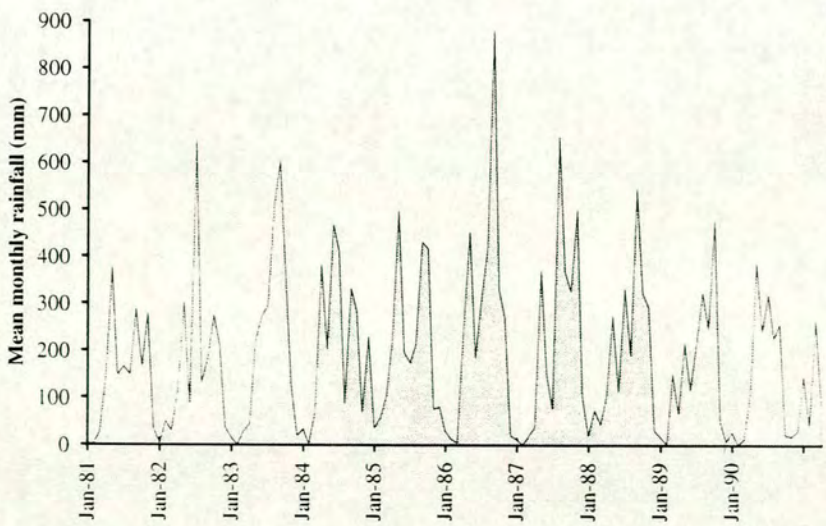


Figure 1.9 Rainfall data from 1981 to 1991.

Coastal waters have temperatures ranging from 26°C to 31°C and relatively constant salinity of 31-34 ‰ throughout the year (Charuchinda and Hylleberg 1984). The region has high seasonal rainfall (averaging approximately 300 mm/month in the wet season over the last 35 years) which transports considerable quantities of fine terrigenous sediment (mainly the clay minerals kaolinite and illite) from the weathered granites and metamorphosed sedimentary rocks of the Phuket Province, into the sea. The inshore areas have highest turbidity, with suspended terrigenous solids frequently as high as 40 mg/l. The waters around the offshore corals (20 + km) commonly have less than 1 mg/l suspended terrigenous solids (Scoffin *et al* 1992). However, these offshore reefs tend to be more exposed to wave action.

1.4.2 The hydrodynamics in Phangnga Bay: The hydrodynamics in Phangnga Bay are influenced by the interaction of terrestrial runoff, tidal currents, monsoon airflow and bathymetry. Work by Siripong *et al* (1987) and Khokiattiwong *et al* (1991) has suggested that much of the variation in the hydrodynamics is due to the monsoons. During the SW Monsoon, (April to November) airflow from the south west causes net surface currents to flow towards the north of the bay (Siripong *et al* 1987). During the NE monsoon (November to April), winds from the north east cause net surface flow to be directed southwards.

1.4.3 The sample sites: Although this thesis used material collected from a number of different sample sites (much of this material was collected by Scoffin and Tudhope on previous visits), the majority of samples used in this thesis were collected from three sites, Tin Smelter, Porites Bay and Aquarium reefs (see Figure 1.7 on page 12). These inshore fringing reefs consist of 100-150 m wide inter-tidal reef flats with near-vertical reef slopes leading down to muddy fore-reef sediments at a depth of 3-4 m (relative to Mean Low Water Springs tide level) (see Figure 1.8 on page 13). While the reef front is dominated by large colonies of *Porites lutea* up to 4 m in diameter, the reef flat can be divided into several zones in which certain species of coral dominate. *Goniastrea retiformis* commonly dominates reef flat areas near the reef front whereas *Goniastrea aspera* dominates reef flat areas closer to the shore. These three sites provide an ideal area in which to study the effects of the monsoons as the Tin Smelter and Porites Bay reefs are on the north-east shore of a peninsula (and are therefore exposed to the NE monsoon) whereas the Aquarium reef is on the south-west shore and feels the effect of the SW monsoon.

1.5 CORAL COLLECTION, CLEANING AND SELECTION FOR ANALYSIS:

1.5.1 Collection: As *Porites lutea* can grow to over two meters in height and several meters in diameter, it was not possible to sample an entire colony. Large cores, one of which was used in this thesis courtesy of Tudhope, were obtained using a specially adapted underwater drill. All the other samples used in this thesis were obtained from coral heads collected from a water depth of 3-4 m below mean low water using a hammer and chisel. In the field, the following coral heads were avoided:

(a) **Coral heads with bumpy surfaces**: Corals with undulating outer surfaces often have uneven fluorescent banding patterns as shown in Figure 1.10. As these bands were difficult to sample (i.e. to cut out), coral heads with bumpy surfaces were avoided.

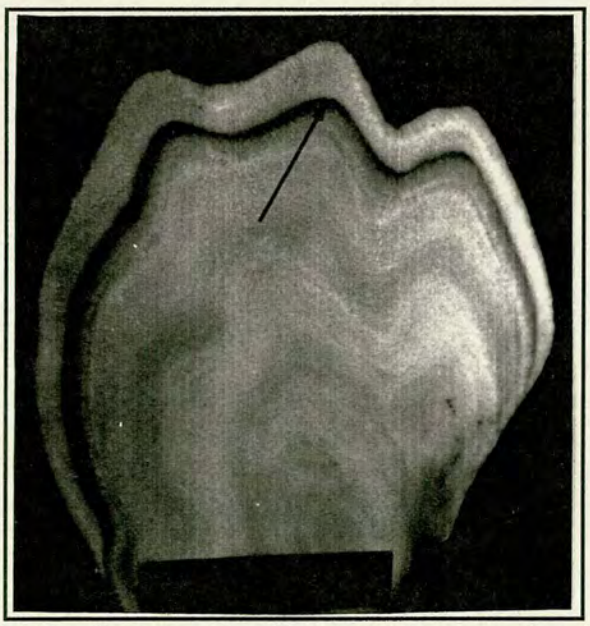
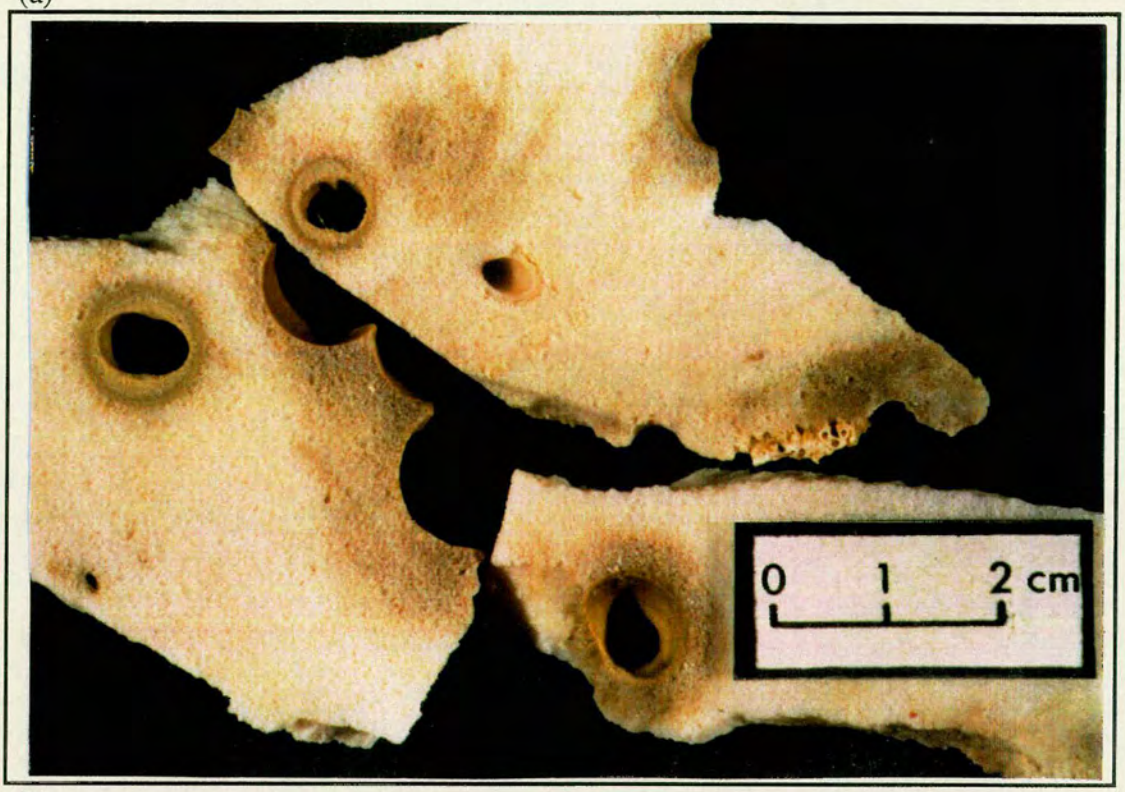


Figure 1.10 Wavy/undulating fluorescent banding due to bumpy surface (coral AQ-15-87). The scale bar is 5 cm long. The dark line (indicated by an arrow) is alizarin stain, put there by Tudhope in November 1986.

(b) **Coral heads with borings**: Large scale (macro) borings are often seen at the surface of a coral. When these corals are cut in half, the borings are quite often surrounded by a brown halo (the nature of which is discussed in Chapter 3, section 3.3, page 55) while the rest of the skeleton is white (see Figure 1.11). As these bored areas do not fluoresce when excited with u/v light (~370 nm), unbored coral heads were chosen.

(a)



(b)

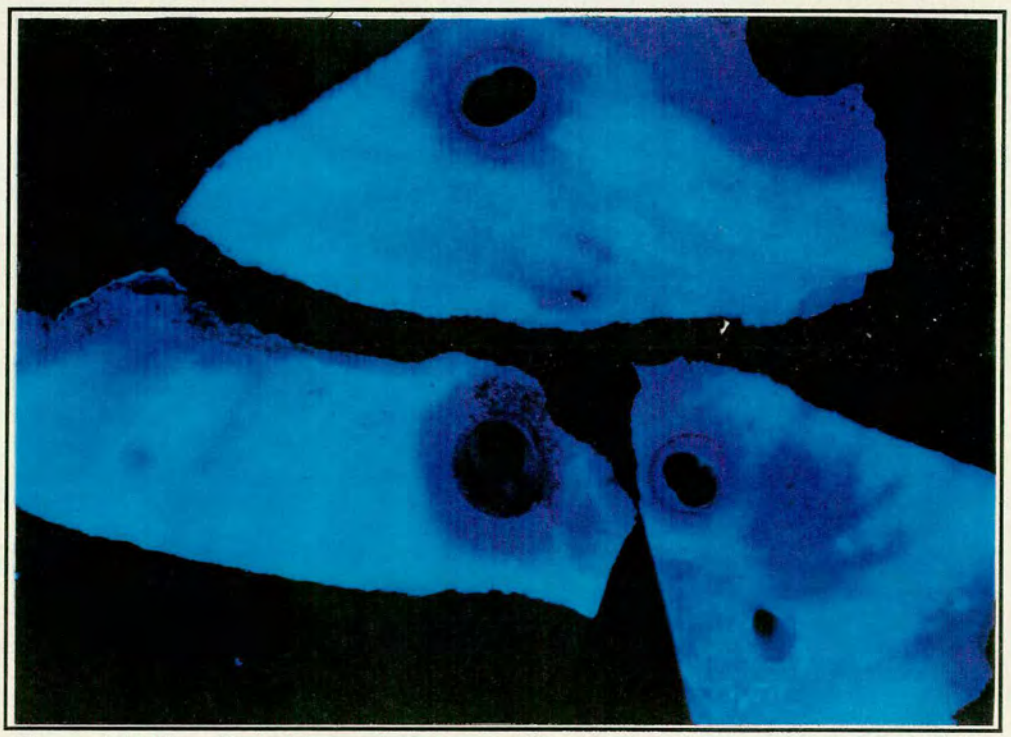


Figure 1.11 The effect of (macro) borings on fluorescence. (a) normal light (b) u/v light.

(c) **Coral heads that were flat or disc shaped**: Rounder heads are commonly found growing on the upper surface of the colony while flatter disc-like heads are more commonly found on the sides and near the base. As linear extension rate generally decreases away from the upper surface, fluorescent bands in the flatter disc shaped head tend to be very narrow which makes them difficult to sample.

1.5.2 Cleaning: When *Porites lutea* was collected, a brown polyp tissue layer between 5-10 mm thick covered its outer surface. As well as imparting a fluorescent signal of its own, this non-structurally bound organic material masks the fluorescence of the skeleton beneath (see Figure 1.12). It was therefore vitally important to remove this, and any other non-structurally bound organic material, from the skeleton. Traditional methods have included soaking in hydrogen peroxide (Scherer and Seitz 1980), sodium hypochlorite (NaOCl) (Veeh and Turekian 1968) or sodium hydroxide (Young 1971). However, none of these procedures were entirely successful as they failed to remove all the organic material, and in some cases they even altered the chemical composition of the aragonite.

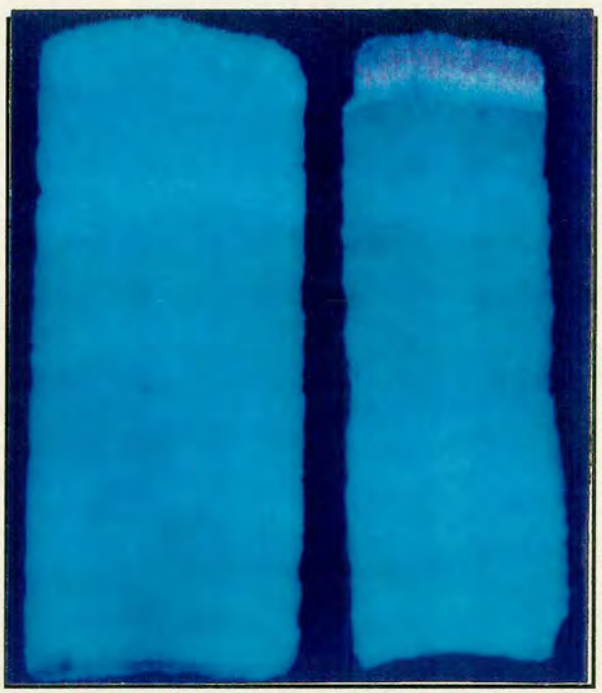


Figure 1.12 Coral skeleton that has been cleaned with NaOCl (right) and coral skeleton that has not been cleaned with NaOCl (left). The living coral surface appears as a pink/orange layer in the uncleaned sample. Samples are approximately 6 cm long.

Organic material can also be removed by heating powdered coral at 550°C (Dean 1974). Although this does not alter the bulk composition of aragonite (it causes an inversion of biogenic aragonite to calcite), it leaves behind an ash residue (Gaffey *et al* 1991). Hydrogen peroxide (H_2O_2) was not used because of its inability to remove polysaccharides (Gaffey and Bronnimann, 1993). In addition to which, with a measured apparent pH of approximately 4.2 for a 30% solution open to atmospheric CO_2, H_2O_2 results in the dissolution of aragonite (Pingitore *et al* 1993, Gaffey and Bronnimann 1993). A plasma asher, which utilises highly reactive oxygen free radicals to oxidise surface organics, was also tested. However, results were disappointing and it was therefore not used.

Harsher or multiple chemical treatments undoubtedly remove more organic material but are also likely to cause greater alteration of the aragonite. Work by Gaffey and Bronnimann (1993) demonstrated that organic material is very effectively removed by full strength (5%) NaOCl and, because of its high pH (11.4), it strongly inhibits the dissolution of calcium carbonate. Although Quale (1991) reported a slight increase in fluorescent intensity after cleaning, no such increase was observed by this author. For these reasons, NaOCl was used to clean the coral in this thesis.

In the interests of hygiene, corals were soaked in a dilute solution of NaOCl (*ca.* 3-4%, a stronger solution was not available) for 48 hours after collection and briefly rinsed in tap water before being shipped back to the UK. Once in the UK, the corals were soaked in (5%) NaOCl for 24 hours. The ability of NaOCl to remove surface organic matter depends on the thickness and bulk density of the sample being cleaned. The thicker the sample and the higher its bulk density, the less effectively NaOCl penetrates the coral skeleton. To avoid this problem, the corals were cut into slices that were approximately 10 mm thick. Although NaOCl is basically non-fluorescent, it absorbs strongly between 300 nm and 420 nm, and weakly between 420 nm and 620 nm (see Figure 1.13). As fluorescent banding in *Porites lutea* is a phenomenon that is seen in the visible part of the electromagnetic spectrum, any residual NaOCl, left on the coral after the removal of non-structurally bound organic material, could potentially absorb (quench) some, or all, of the fluorescence emitted by the coral fluorophores and thus prevent coral fluorescence from being seen.

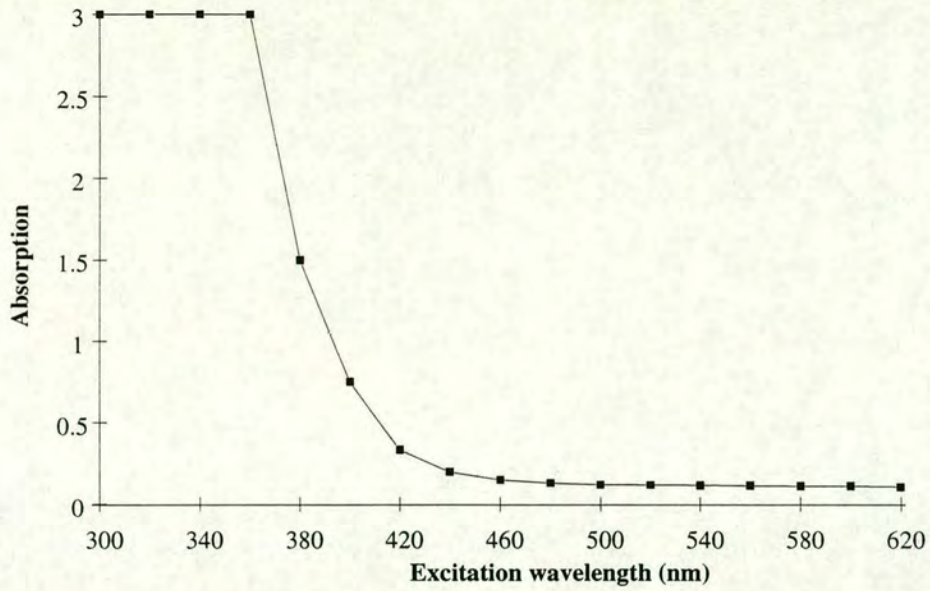


Figure 1.13 The absorption spectrum of NaOCl.

In addition, results in Chapter 4, Figure 4.6, page 79, and Chapter 6, Figure 6.2, page 171, show that coral, both in solution and in the solid state, have two main excitation peaks, one at 330-340 nm and the other at 390 nm. Figure 1.13 demonstrates that NaOCl absorbs strongly in this region and therefore, not only does NaOCl have the ability to quench coral fluorescent emissions, it could also prevent coral fluorophores from being excited. Thus, as much of the NaOCl should be removed from the skeleton as possible. As various authors have reported carbonate dissolution when coral was soaked in distilled water (DH_2O) (Amiel *et al* 1973; Pingitore *et al* 1993), the corals were rinsed with tapwater. A full week of rinsing was found to be essential for complete removal of NaOCl.

As the cleaning process does not appear to significantly affect coral fluorescence, it would appear that the skeleton offers some protection to the fluorophores. Indeed, when the skeleton was dissolved using HCl, and NaOCl was added to the resulting solutions, coral fluorescence was greatly reduced (see Figure 1.14). This is therefore another reason why NaOCl should be removed.

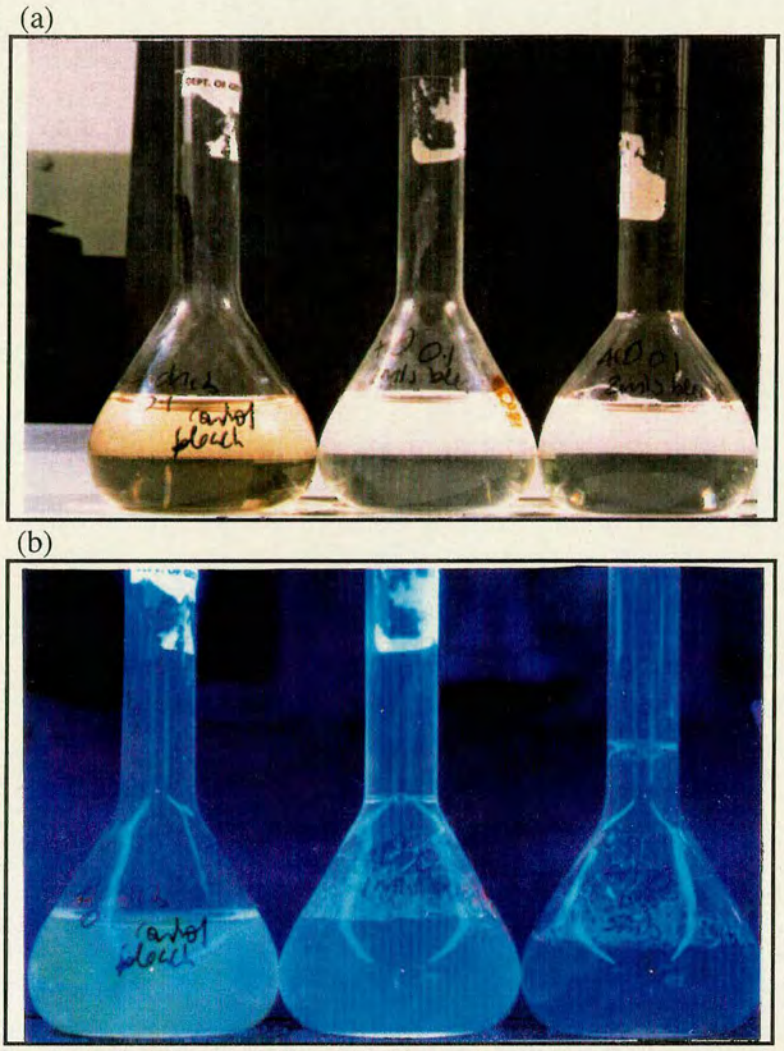


Figure 1.14 The effect of adding NaOCl to Aldrich humic acid seen in normal and u/v light. The photographs were taken 60 minutes after the addition of NaOCl. The concentration of Aldrich humic acid was constant in all flasks. From left to right, 20 mls of Aldrich humic acid control, the effect of 1 ml of NaOCl, the effect of 2 mls of NaOCl. (a) normal light, (b) u/v light. It can be seen that 60 minutes after the addition of NaOCl, both the colour in normal light and fluorescence appear to have been quenched.

1.5.3 Selection for analysis: Bright and dull fluorescent bands were identified under a u/v lamp (Phillips TLD 18w/08). Although fluorescent banding in *Porites lutea* is described in this thesis in terms of bright and dull bands, these are end members in a continual spectrum. There are, in fact, many parts of the coral skeleton that can only be described as being transitional (i.e. are neither bright or dull but somewhere in between). For the vast majority of work in this thesis, these transitional areas were not sampled. In addition, the following parts of the coral skeleton, except where stated, were also avoided:

(a) **Living coral surface**: Even though both bright and dull bands have been identified at the living coral surface in some samples (see Figure 1.15 for dull band at the living coral surface and Figure 1.5a and b on page 10 for bright bands at the living coral surface), it is generally very difficult to determine band type in this region of the skeleton. For this reason, this part of the skeleton was not sampled (except where otherwise stated). Exactly why fluorescent banding is difficult to determine at the living coral surface is unknown, but it could be due to polyp tissue that is not completely removed during the cleaning process.

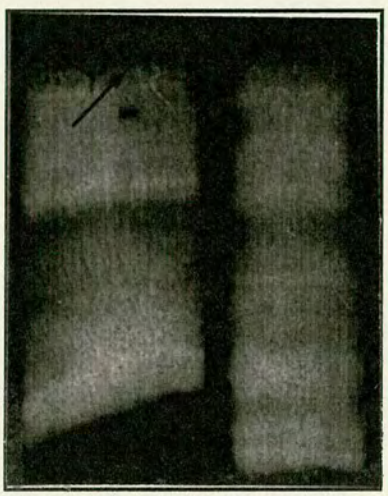


Figure 1.15 Dull band at the living coral surface. These corals were collected in August 1993 from the Tin Smelter Reef. An arrow indicates the position of the dull band at the living coral surface.

(b) **Discoloured brown areas**: As the brown discoloured areas associated with macroborings do not fluoresce when excited with u/v light (see Figure 1.11), these areas were avoided.

(c) **Anchorage points:** As these areas tend to be heavily cemented, they would affect measurements of bulk density and porosity. As both of bulk density and porosity have been shown to affect coral fluorescence (see Chapter 2), these areas were therefore avoided.

1.6 LAYOUT OF THIS THESIS:

Work in this thesis begins with the study of coral fluorescence in the solid state to asses the effects of porosity and microboring activity on the colour and intensity of coral fluorescence (Chapters 2 and 3 respectively). The fluorophores thought to be responsible for coral fluorescence are extracted from the skeleton in Chapter 4 to enable the effects of pH, concentration and the presence of certain metal ions, to be studied. Using a fluorescence microscope, the spatial distribution of fluorophores through the skeleton at the microscopic level is examined in Chapter 5. Work then concentrates on the measurement of solid state fluorescence (Chapter 6) and the possible reason(s) why bright fluorescent bands are deposited during the dry season in the Thai area (Chapter 7). The thesis finishes with a summary of the principal conclusions (Chapter 8).

CHAPTER 2. THE RELATIONSHIP BETWEEN FLUORESCENT BANDING AND PORE SPACES

CHAPTER 2. THE RELATIONSHIP BETWEEN CORAL FLUORESCENCE AND PORE SPACES

2.1 INTRODUCTION:

The most logical first step in attempting to discover the cause or causes of fluorescent banding in *Porites lutea* is the examination of solid coral under a u/v lamp. When coral skeleton is excited with longwave u/v light (~370 nm), it appears that fluorescent emissions from the pore spaces (i.e. the gaps between skeletal material) are more intense, and possibly even a different colour, to those from the upper surface, irrespective of band type (see Figure 2.1).

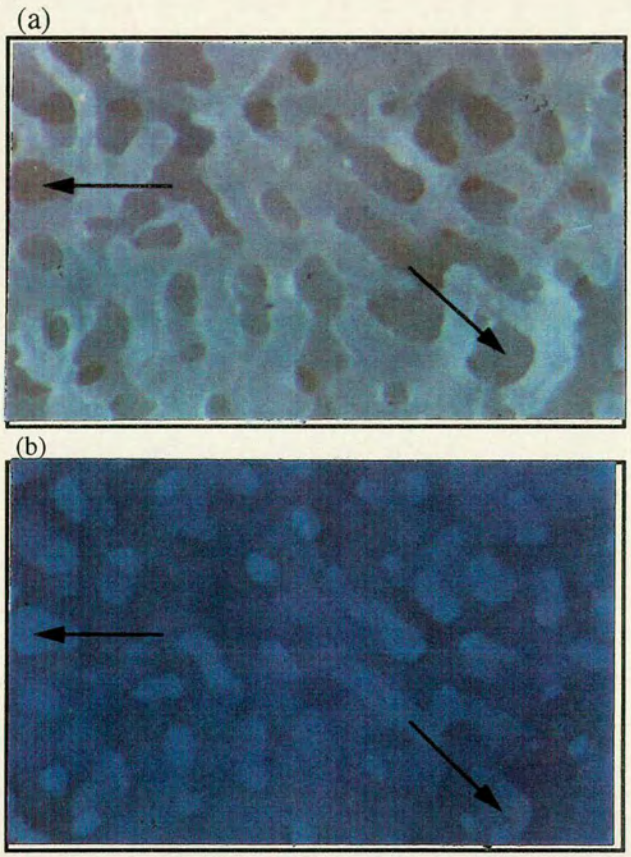


Figure 2.1 Coral surface seen in (a) normal light and (b) u/v light, using a binocular microscope. The arrows indicate the position of a couple of pore spaces (there are of course many more in the above sample which have not been marked). At a magnification of x25, the width of view is 0.32 cm.

These observations suggests that there is a relationship between coral fluorescence and porosity. As porosity is related to bulk density (BD), by the following formula:

$$BD = \frac{2.94 \times (100 - \%PS)}{100} \quad (1)$$

where:

2.94 = the density of aragonite (g/cm^3)
%PS = % porosity

It would appear that a relationship may also exist between coral bulk density and fluorescence.

2.1.1 Objectives and layout of this chapter: The aim of this chapter is to determine whether there is a relationship between fluorescent banding and porosity (or bulk density) in corals of the species *Porites lutea* from Ko Phuket. Work in this chapter has been divided into three sections. The first section involves the examination of density x-radiograms of corals collected from a number of different reefs in the study area. The second section deals with the direct measurement of bulk density and porosity from coral blocks and thin sections respectively. The final section concentrates on the effects of artificially enhancing and reducing porosity/bulk density.

2.2 COMPARING BULK DENSITY X-RADIOGRAMS WITH FLUORESCENT IMAGES:

The easiest way to determine whether bulk density and fluorescent banding are related was to compare density x-radiographs with photographs taken under u/v light from the same sample.

2.2.1 Background: The study of sclerochronology began in the early 1970's when density banding was detected in massive coral skeletons by x-radiography (Knutson *et al* 1972, Buddemeier *et al* 1974). Areas of relatively high skeletal density appear as dark bands and areas of relatively low skeletal density appear as light bands. Knutson *et al* (1972) suggested that a pair of bands (a high/low density band couplet) represented a year's growth; a hypothesis that was subsequently confirmed by staining experiments (Wellington and Glynn, 1983). However, Charuchinda (1985)

has reported that in one year, four skeletal bands of differing density were deposited in some *Porites lutea* from Ko Phuket.

It is 'generally believed' (Highsmith 1979) that low density bands are produced under optimum growth conditions and high density bands are produced under non-optimum growth conditions. However, the exact cause of the density banding is not known for certain as the following factors are all thought to have an effect: light, water temperature, levels of suspended sediment, salinity, water agitation, nutrient availability, reproductive activity (the production of gametes may reduce the energy available for growth), and the roles played by symbiotic zooxanthellae in influencing calcification and endolithic algae in modifying density patterns. It is therefore reasonable to assume that optimum conditions for coral growth in one location may not be the same as those in another, even for corals of the same species.

2.2.2 Methods and materials: For reasons given later, samples were cut as evenly as possible to a thickness of 5 mm using a diamond tipped saw. As coral fluorescence is basically a surface phenomenon (see section 2.4, pages 34-36), fluorescent images relate to the surface being irradiated. This means that fluorescent images from different sides of the same coral block may vary depending on the three dimensional nature of the banding. For this reason, both sides of the slab were photographed under u/v light (emission maxima 370 nm). A u/v cut-off filter was used to prevent u/v light from the lamp reflecting off the coral surface and being recorded on film. The samples were then photographed using an x-ray machine with Kodak type AX film at exposures of approximately 120 s at 70 kV and 3 mA. Each x-radiograph (life-size print prepared from contact negative) was spliced against the life-size u/v print of the same coral allowing direct comparison of skeletal density and fluorescent banding for each sample.

2.2.3 Results and discussion: In *Porites lutea* from Papua New Guinea and Indonesia, Scoffin *et al* (1989) noticed that where bright and dull band fluorescent couplets and dense/less dense couplets were clearly visible (60% of coral specimens), bright bands correlated with less dense bands in the skeleton. In Ko Phuket, however, bright bands appear to correlate with less dense bands in corals from the Aquarium reef and with more dense bands in corals from the Tin Smelter and Porites Bay reefs (see Figure 2.2).

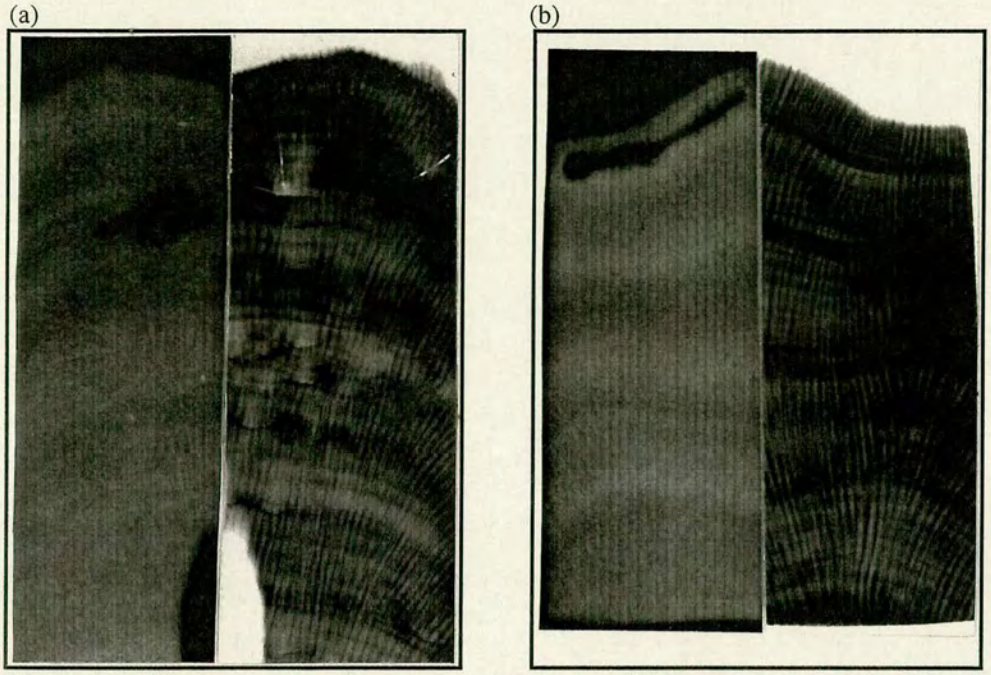


Figure 2.2 Fluorescent banding versus density banding. For each sample (actual size), the fluorescent image is on the left and the x-radiogram on the right. (a) AQ-4-87 in which bright fluorescent bands appear to correlate with less dense bands, (b) PB-8-87 in which bright bands appear to correlate with more dense bands.

There are, however, a number of problems associated with density x-radiographs which may account for the large number of corals in which bright fluorescent bands correlated with both dense and less dense bands (see Figure 2.3).

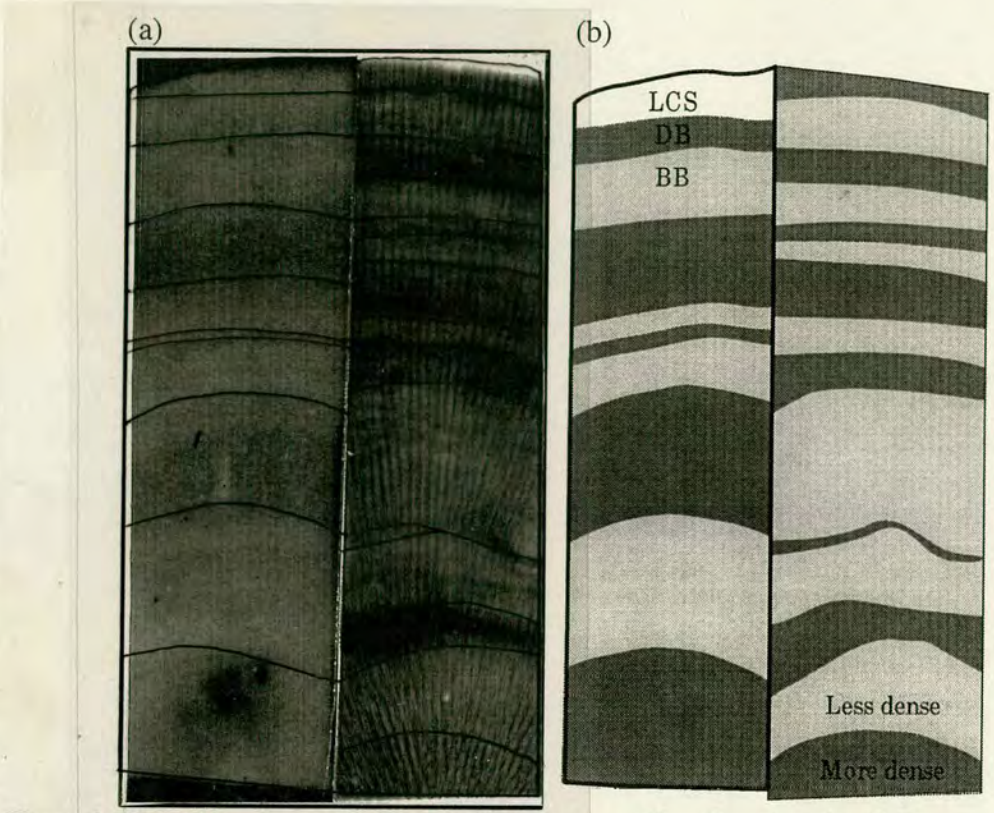


Figure 2.3 Bright bands correlating with both dense and less dense bands. (a) Fluorescent image on the left and density x-radiogram on the right for TS-9-87 (actual size); (b) simplified schematic representation of fluorescence and density banding showing that bright bands correlate with both dense and less dense bands. BB = bright band, DB = dull band, LCS = living coral surface.

Barnes and Lough (1989) acknowledged that x-ray images are two dimensional (i.e. the thickness of the skeletal slice is effectively infinitely compressed). Thus, any particular density band seen in x-radiographs represents the average absorption of x-ray energy through that part of the slice rather than from the surface alone. This means that artificial density bands may be produced as a result of the following:

(a) **Areas of varying thickness:** For example, if a block of coral with a uniform bulk density was cut such that one end was thicker than the other, the thicker end would appear to be denser. This is why blocks were cut as evenly as possible to a uniform thickness.

(b) **Changes in the orientation of skeletal elements**: For example, if a corallite wall was parallel to the x-rays it would produce a thin dense band. The same corallite wall would produce a thicker but less dense band at any other angle, the thickest and least dense band being produced when the corallite wall was perpendicular to the x-ray beam.

To summarise, although it seems unlikely that problems inherent in the production of x-radiographs could produce such contrasting bulk density results in the same species of coral from different sides of a peninsula, the possibility can not be discounted. Therefore, photographic evidence of this nature should only be used to supplement bulk density measurements obtained directly from coral blocks.

2.3 THE DETERMINATION OF BULK DENSITY FROM CORAL BLOCKS:

Although considerable work has been done on the bulk density of *Porites lutea* from and around Ko Phuket (Scoffin *et al* 1991), measurements were taken from coral blocks that contained two fluorescent couplets. No attempt has been made to calculate the bulk density of individual bright and dull bands.

2.3.1 Methods and materials: For sample selection and preparation see Chapter 1 section 1.5, pages 15-21. All samples were taken from reef front corals as conditions across the reef (i.e. reef front to reef flat) are unlikely to be uniform. Work by Allison (1994) has suggested that bulk density varies across the surface of the coral as a consequence of differential linear growth rates, with higher bulk densities occurring where the linear growth rate is slowest. Thus, in order to compare the bulk density of bright and dull bands effectively, samples must be taken from the same region of the coral. The axis of maximum linear extension was chosen as fluorescent bands are wider here than in any other part of the coral thus making it easier to obtain large samples (the importance of this is discussed later in this section).

Coral skeleton was cut into blocks with right angled corners using a diamond tipped saw. Cubes were cut wherever possible although certain fluorescent bands were too thin to allow this, in which case cuboids were cut. The blocks were then jet washed, placed in a beaker of distilled water (DH_2O) and sonicated for 99 minutes at 30°C. These two stages were done to remove coral powder generated during the cutting process. Evidence suggests that unless jet washing is extremely thorough, coral dust can still be detected in the pore spaces. The presence of coral powder in a block would increase its mass and thus make its bulk density larger than it should be. This has more serious implications for smaller blocks where the dust to skeletal material ratio is likely to be more significant. Once cleaned, the blocks were placed in an oven at 100-105°C for an hour as the complete removal of trapped water was essential if reliable bulk density measurements were to be obtained. The blocks were

then allowed to cool to room temperature in a dessicator (to prevent the adsorption of water from the atmosphere) before any measurements were made. The samples were weighed before their X, Y and Z dimensions (length, height and width) were measured using vernier callipers. Each dimension represents the mean of four independent measurements (i.e. a measurement at each corner). It was important to weigh the blocks before the X, Y and Z dimensions were measured as the measuring process often resulted in the slight loss of skeletal material. In order to determine the error associated with measurements of bulk density, one large block of coral was weighed eight times and its X, Y and Z dimensions measured eight times. This block was then cut in half and the process repeated (see Appendix A2.1). Results are presented in Figure 2.4 and show that although the errors associated with the estimates of bulk density are low, they increase with decreasing sample mass. In addition, and perhaps more importantly, bulk density appears to decrease with decreasing sample mass.

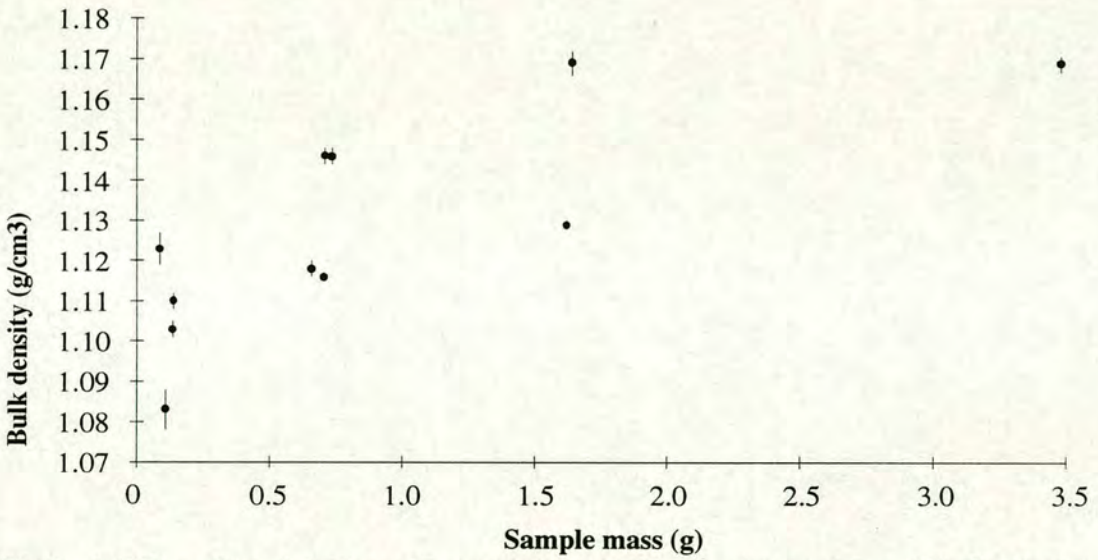


Figure 2.4 The effect that decreasing sample mass has on the precision of bulk density measurements made from PB-4-87. The error bars represent the 99% confidence interval. Although eight samples should have been produced whose mass was less than 0.25 g, four of the eight were not considered suitable on account of their shape. The increasing spread with decreasing sample mass could have been due to the following: (a) the fact that no effort was made to obtain blocks that were fluorescent band specific i.e. the starting block may not have had a uniform bulk density, (b) the smaller the sample the higher its chances of being dominated by either pore space or skeletal material increase, and (c) bulk density is not homogeneous throughout a band.

This apparent decrease in bulk density with decreasing sample mass was thought to be due to the loss of material from the outer 1 mm of the coral block as a consequence of the cutting process. This process is referred to in this chapter as 'flaking' and is more likely to affect smaller blocks as their surface area to volume

ratio is greater than in larger blocks. In order to determine whether the observed decrease in bulk density with decreasing sample mass is significant, a Students 't' test was performed (see Appendix A2.2). Results show that no relationship between sample mass and bulk density exists (even at the 90% confidence level) when samples greater than 0.25 g are used. Thus, to minimise the problems associated with 'flaking', samples greater than 0.25 g were used in this section.

2.3.2 Results and discussion: Bright and dull band bulk density was determined for a number of samples which had been collected from different sides of the peninsula.

Table 2.1 Bright and dull band bulk densities.

Inshore	BB bulk density	Number of samples	DB bulk density	Number of samples	U-Test	Status
NE shore						
AQ-1-88	1.154	9 (5)	1.228	14 (3)	NS	DB>BB
AQ-35-87	1.184	11 (2)	1.162	10 (3)	NS	BB>DB
AQ-1-92	1.173	9 (1)	1.218	8 (1)	MS	DB>BB
SB-1A-88*	1.139	8 (1)	1.174	8 (1)	NS	DB>BB
SB-1-92	1.108	12 (3)	1.183	9 (2)	MS	DB>BB
Mean	**1.151**		**1.184**			**DB>BB**
SW shore						
PB-2-90	0.995	8 (2)	0.967	11 (3)	NS	BB>DB
PB-6-90	0.955	10 (1)	0.888	12 (1)	S	BB>DB
TS-4-87	0.682	13 (1)	0.740	14 (1)	NS	DB>BB
TS-9-87	0.985	6 (3)	0.864	4 (2)	VS	BB>DB
TS-10-88*	1.040	10 (1)	0.942	10 (1)	S	BB>DB
Mean	**0.931**		**0.880**			**BB>DB**

Key: * = includes samples that are below the 0.25 g cut-off value because of a shortage of sample material. U-Test = Mann-Whitney U-Test; NS = not significant; MS = marginally significant at the 90% confidence level; S = significant at the 95% confidence level; VS = very significant at the 99% confidence level; BD = bulk density; BB = bright band and DB = dull band; AQ = Aquarium reef; PB = Porites Bay reef; SB = Shark Bay reef; TS = Tin Smelter reef. The number of samples measured and the number of bands used (in brackets) are also indicated.

Results in Table 2.1 show that bright band bulk densities are generally higher than dull band bulk densities in *Porites lutea* from Tin Smelter and Porites Bay Reefs but lower in Aquarium and Shark Bay Reefs. A similar relationship between porosity and coral fluorescence was obtained (see Appendix 2.3 for further details). This was to be expected considering that bulk density and porosity are inversely related (see equation 1 on page 25). Thus, as the relationship between bulk density and fluorescent band type varies depending on what side of the peninsula the corals are on (which could suggest a monsoonal control on bulk density), it would appear that

bulk density is not the major control on fluorescent banding thus confirming the photographic based observations made in section 2.2.

2.4 ARTIFICIALLY CHANGING POROSITY:

Although evidence in sections 2.2 and 2.3 has shown that bulk density/porosity are not directly responsible for fluorescent banding, Figure 2.1 on page 24 still suggests that porosity has some effect on coral fluorescence. Thus, the porosity of the skeleton was artificially changed to see what effect this had on coral fluorescence.

2.4.1 Methods and materials: Porosity was artificially reduced by crushing the coral skeleton with a pestle and mortar. The coral powder was sieved into three fractions; 150 μm to 355 μm, 40 μm to 150 μm, and less than 40 μm. Equal amounts of material were weighed out and pressed into pellets. Fluorescent intensity was recorded using the solid fluorescence measuring device developed in Chapter 6 (see section 6.3, pages 175-188). The pellets were excited at 365 nm and fluorescence was recorded at 450 nm. In addition, porosity was artificially enhanced by drilling holes and cutting grooves in the surface of the skeleton using a diamond tipped saw, and by adding drops of concentrated HCl to localised areas.

2.4.2 Results and discussion:

The effect on fluorescent intensity: Results suggest that artificially reducing porosity by crushing reduces fluorescent intensity (see Table 2.2).

Measurement	150-355 μm	40-150 μm	Under 40 μm
1	520	364	253
2	459	350	253
3	508	414	244
4	486	343	260
5	459	351	286
6	482	335	350
7	590	382	269
8	492	378	253
9	523	360	276
10	592	343	241
Mean	**511.1**	**362**	**268.5**
STDEV	47.46	23.77	31.89
% error	9.29	6.57	11.88

Table 2.2 The effect on fluorescent intensity of crushing. Key: All values presented in Table 2.2 are in mv as this is how the fluorescence measuring device records fluorescent intensity. Measurements 1 to 10 were taken at random from different positions on the pellet surface. STDEV = standard deviation. % error = STDEV as a percentage of the mean.

Results also show that artificially increasing porosity by drilling holes and cutting grooves appears to increase fluorescent intensity (see Figure 2.5).

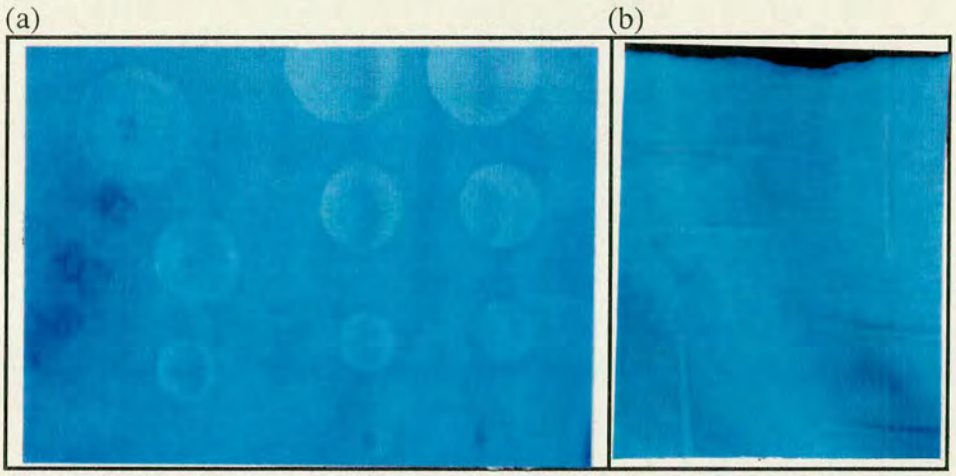


Figure 2.5 The effect of increasing porosity by (a) drilling holes (b) cutting grooves.

Miller (1988) has reported that areas that are mechanically damaged, such as cracks, often become luminescence centres. Thus, the increase in fluorescent intensity seen in Figure 2.5 could have been brought about by some form of lattice-strain effect. However, the fact that fluorescence also increases with chemical enhancement of porosity (see Figure 2.6) suggests that lattice-strain effects were not significant.

Figure 2.6 Adding concentrated HCl to a localised area of coral skeleton (SB-6-88). The acid produced hole is on the left hand side (indicated by the arrow). The other holes were produced by drilling.

One possible explanation that could account for the increases seen in Figures 2.5 and 2.6 relates to the number of fluorophores excited, the greater the number the more intense the fluorescence. The number of fluorophores excited depends on: (1) the depth to which u/v light penetrates the skeleton, and (2) the area irradiated by u/v light.

The depth to which u/v light penetrates coral skeleton was estimated in the following way. All absorption data were obtained using a Unicam 8620 ultra-violet/visible spectrometer. As coral solutions have two main excitation peaks, one between 330-340 nm and the other at 390 nm (see Chapter 4, Figure 4.6, page 79), absorption was measured between 300 nm and 420 nm. Solid coral material was stuck onto a glass slide with epoxy resin (araldite) to enable sub-millimetre thickness sections to be produced. All samples were compared to a glass slide (blank) which had epoxy resin smeared on its surface. Results using *Porites lutea* suggest that u/v light can penetrate aragonite to a depth of between 500 μm and 1000 μm (the depth of u/v penetration depended on bulk density of the coral, the higher the bulk density the lower the depth of u/v penetration). Thus, the corallite walls appear to act as light tubes which allow the u/v light to penetrate to quite large distances (see Highsmith 1981). As a consequence of this, the true depth to which u/v light penetrated solid aragonite was not determined. To overcome this problem, coral skeleton was ground to less than 40 μm and pressed into a pellet. The thinnest pellet that could be made was 1.33 mm thick and although no transmitted light was detected (i.e. all u/v light was either absorbed or reflected), it was still not possible to determine accurately the depth to which u/v light penetrates aragonite. A non-porous form of aragonite was therefore required which could be ground down to less than 1 mm in thickness. A conch shell (composed of aragonite) was used and although it is acknowledged that this is far from ideal (i.e. different lattice structure and organic content), it was hoped that results from this experiment would provide a useful first-order estimate of the depth of u/v penetration in biogenic aragonite. A section of the shell was cut out and stuck onto a glass slide with epoxy resin.

Table 2.3 The amount of u/v light absorbed (%) by non-porous aragonite (conch shell).

Thickness (μm)	330 (nm)	340 (nm)	360 (nm)	365 (nm)	390 (nm)	420 (nm)
35	99.99	96.85	96.20	96.00	95.35	94.50
50	99.99	99.99	99.20	98.85	97.05	94.25
70	99.99	99.99	99.99	99.99	99.99	98.45
90	99.99	99.99	99.99	99.99	99.99	99.97

Values relate to the percentage absorption at the excitation wavelength indicated. The wavelengths displayed in this table were chosen for the following reasons: the main excitation peaks in coral solutions occur between 330-340 nm and at 390 nm (see Chapter 4, Figure 4.6, page 79); 360 nm and 365 nm correspond to the wavelengths that coral fluorescence has commonly been studied at; and 420 nm wavelength was recorded to ascertain whether visible light is absorbed to a greater extent.

Results in Table 2.3 show that the depth to which u/v light penetrates biogenic aragonite varies according to the wavelength of u/v light used, decreasing with decreasing wavelength. The depth of u/v penetration is also likely to vary according to the amount and type of organic material present in the carbonate. However, a full quantification of the effect of this on the colour and/or brightness of fluorescence was beyond the scope of this thesis. This chapter therefore assumes that the depth of u/v penetration in both bright and dull bands is the same. When the conch shell was excited at 365 nm, the estimated depth of u/v penetration was between 50 μm and 70 μm. If it is assumed that corallite walls are cylindrical in cross section and that, as a consequence of multiple reflections, all sides of a corallite wall are excited, then, only when corallite walls are thicker than 100-140 μm will it not be possible to generate fluorescence from all parts of the skeleton when it is excited at 365 nm (see Figure 2.7).

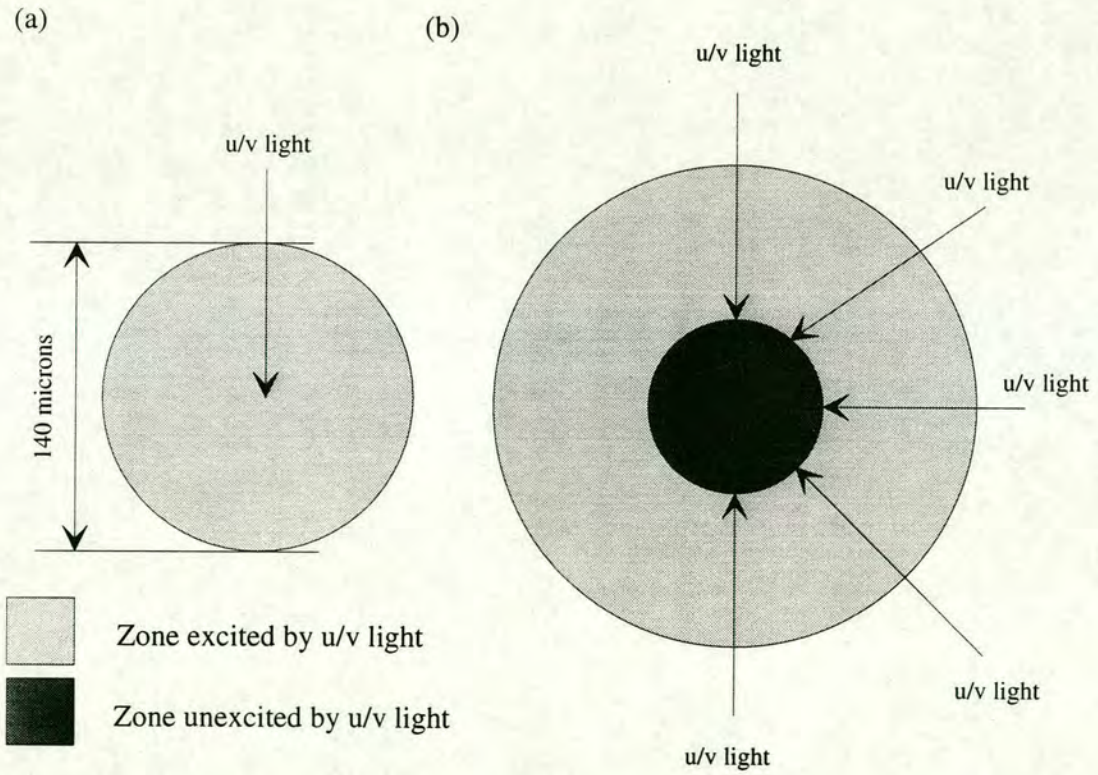


Figure 2.7 Schematic cross section of a corallite wall showing the depth to which 365 nm u/v light can penetrate aragonite. (a) corallite wall diameter = 140 μm, (b) corallite wall diameter = 200 μm. The depth of u/v light penetration is 70 μm in both (a) and (b). Assuming corallite walls are cylindrical in cross section, only when the diameter of the corallite wall is greater than 140 μm will it not be possible to generate fluorescence from all parts of the skeleton.

Corallite wall thickness in *Porites lutea* generally varies between 50 μm and 150 μm although some of the thickest parts are up to 300 μm (see Figure 2.8). Thus,

assuming the depth of u/v penetration in *Porites lutea* is similar to that in the conch shell, fluorescence could be generated from most parts of the skeleton when excited at 365 nm.

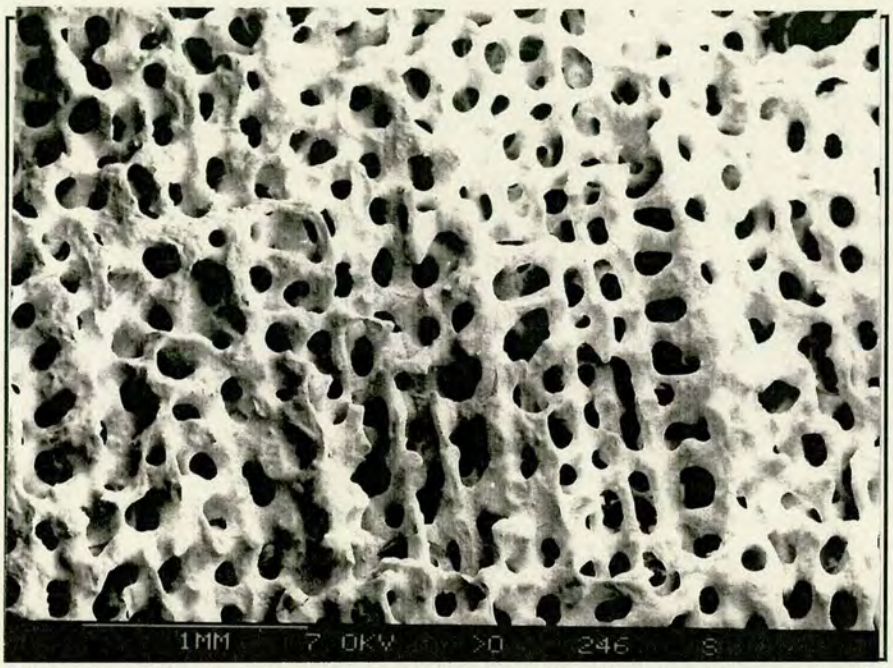


Figure 2.8 A section of Porites lutea skeleton below the *Ostreobium* band (green algal band) seen using a scanning electron microscope. This sample was collected from Porites Bay and was cleaned in the manner described in Chapter 1, section 1.5.2 (page 17-19) before examination.

However, results in Table 2.3 (page 34) suggest that even when the conch shell was only 35 μm thick, very little u/v light (~5%) passed all the way through the skeleton. Exactly how much of the remaining 95% was absorbed or reflected off the conch surface was not determined. This, therefore, suggests that the bulk of the u/v light that penetrates the aragonite (i.e. is not reflected/scattered away) is absorbed in the outer few microns. In other words, results in this section suggest that coral fluorescence is probably a surface phenomenon. Thus, the greater the surface area excited, the greater the fluorescent intensity.

If porosity/surface area does have an effect on coral fluorescence, perhaps it could explain why *Goniastrea retiformis* and *Platygyra daedalea*, two commonly found species of coral on the Thai reefs, are more fluorescent than *Porites lutea* (see Figure 2.9). Bulk density was determined using blocks cut from *Porites lutea*, *Goniastrea aspera* and *Platygyra daedalea* collected from Tin Smelter Reef. The figures in Table 2.4 were provided by Scoffin and are unpublished. They were determined in the manner described in section 2.3.1 using blocks of coral with dimensions greater than 10x10x10 cm. Such large blocks were required for the following reasons: (1) to

minimise the effects of 'flaking', and (2) to ensure that samples contained a high number of corallites. If the size of the block is around the same size as the diameter of a single corallite, the chances of a non-representative bulk density measurement being produced are high. This is because the block could be dominated by either pore space (underestimating bulk density) or skeletal material (overestimating bulk density). Thus large samples were taken to avoid this problem.

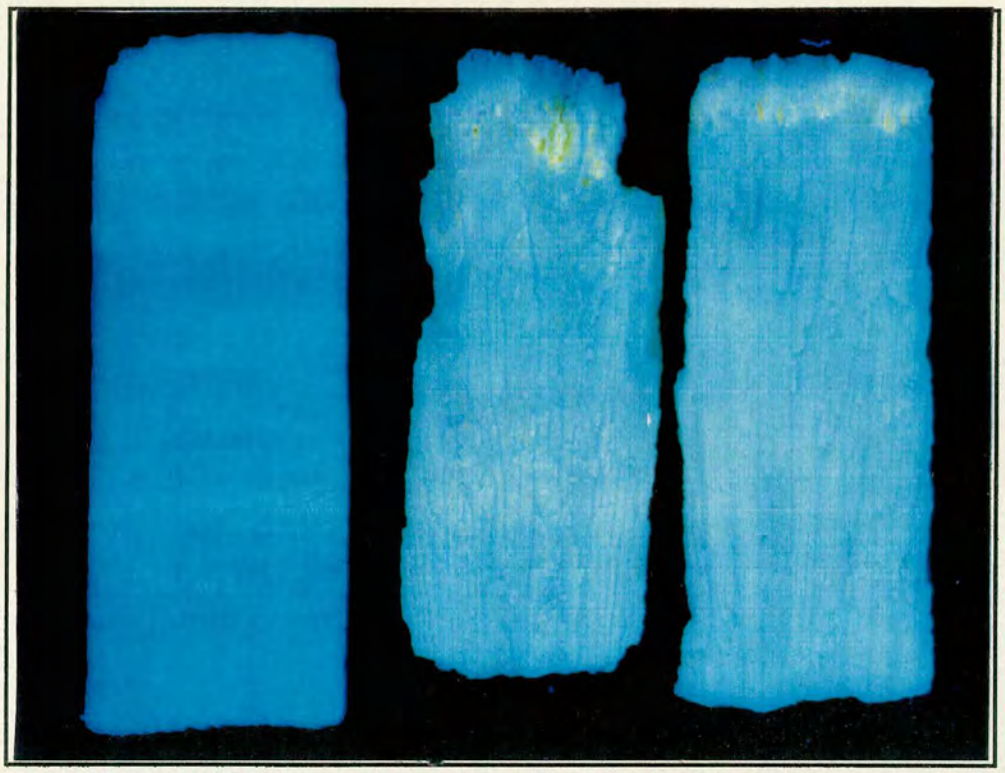


Figure 2.9 Fluorescent banding in *Porites lutea, Platygyra daedalea* and *Goniastrea retiformis* from Porites Bay Reef under u/v light. From left to right: *Porites lutea, Platygyra daedalea* and *Goniastrea retiformis*.

Table 2.4 The average bulk density of *Porites lutea, Platygyra daedalea and Goniastrea retiformis*.

Species	Bulk density	(n)
Porites lutea	1.04	10
Platygyra daedalea	1.11	8
Goniastrea retiformis	1.19	6

Key: (n) = the number of samples used.

Although the mean bulk density of these three species of coral are probably all within error (errors were not provided by Scoffin), results show that *Porites lutea* has the lowest bulk density (i.e. it has the highest porosity). If corallite ornamentation

was identical in these species, then the amount of skeletal material that would be excited by u/v light should be higher in *Porites lutea* than in the other species. In other words, *Porites lutea* should be the most fluorescent. This is clearly not the case and could be due to differences in corallite ornamentation between the species. Figure 2.10 shows the corallite ornamentation in *Porites lutea* and *Goniastrea retiformis*.

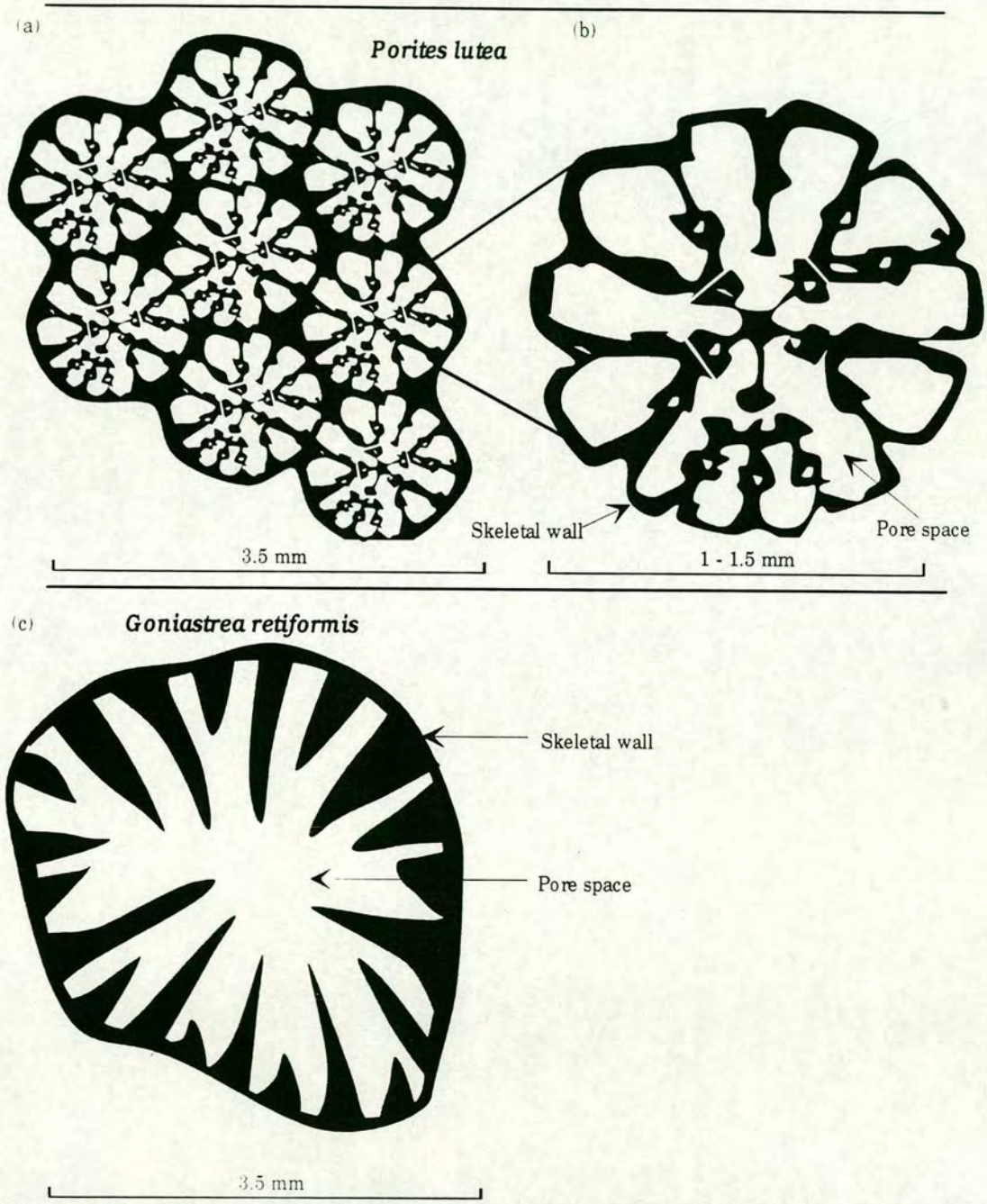


Figure 2.10 Cross sections of *Porites lutea* and *Goniastrea retiformis* showing corallite ornamentation. (a) 8 *Porites lutea corallites*, (b) 1 *Porites lutea* corallite expanded, (c) 1 *Goniastrea retiformis corallite*.

The surface area of the two types of coral was estimated by measuring the length of a line that traces the corallite wall from plan view images (see Figure 2.10). This line was 30.5 mm long in *Goniastrea retiformis* and 14.4 mm long in *Porites lutea*. These values were determined assuming the corallite diameter was 3.5 mm in *Goniastrea retiformis* and 1.25 mm in *Porites lutea* (values from Veron 1986). Although a single *Goniastrea retiformis* corallite has a larger surface area than a single *Porites lutea* corallite, the latter is much smaller than the former. Calculations suggest that there are approximately 7.8 *Porites lutea* corallites to one *Goniastrea retiformis* corallite. 14.4 mm was therefore multiplied by 7.8 to give 112 mm. These results suggest that in an equal area of coral, a larger amount of skeletal material would be exposed to u/v light in *Porites lutea* (112 mm) than in *Goniastrea retiformis* (30.5 mm). Thus, it would appear that the amount of skeletal material exposed to the u/v excitation beam is probably not the main reason why *Porites lutea* was the least fluorescent coral species examined. The difference in fluorescent characteristics between the different species is more likely to be due to the concentration and/or type of fluorophores contained within each species. Using the method described in Chapter 4, section 4.2.5 (see page 70) for the dissolution of the skeleton and the production of a coral solution, results suggest that the concentration of fluorophores/g of coral is higher in *Goniastrea retiformis* than in *Porites lutea* (see Figure 2.11). In other words, the difference in fluorescent intensity seen in Figure 2.9 (page 37) between *Porites lutea* and *Goniastrea retiformis* is probably due to the concentration of fluorophores/g of coral and not surface area differences.

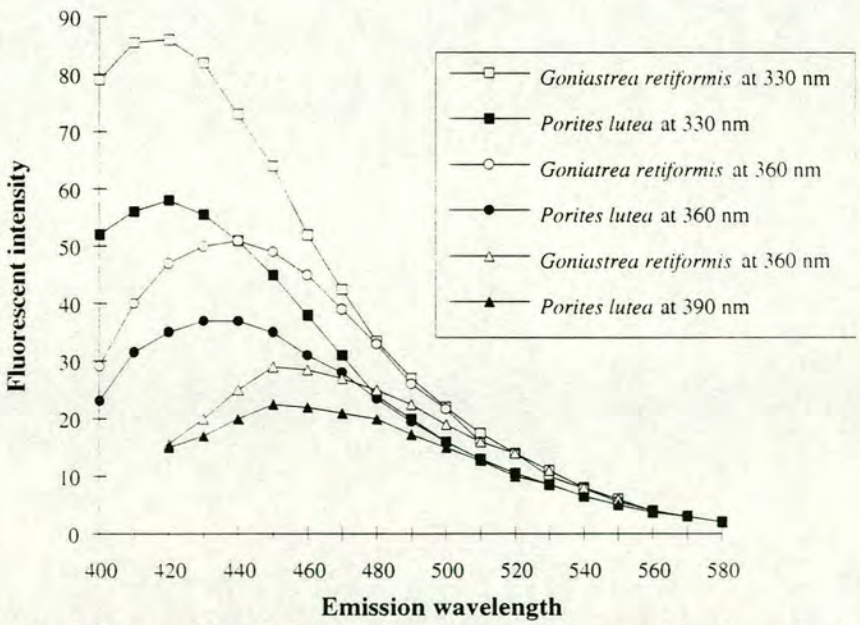


Figure 2.11 Emission profiles of *Porites lutea* and *Goniastrea retiformis* at three excitation wavelengths.

To summarise, although bulk density/porosity does not appear to be the main cause of fluorescent banding, it does appear to have some effect on fluorescent intensity. The more porous the skeleton, the brighter the fluorescence. However, observations made from several different species of coral suggest that bulk density/porosity has less of an effect on fluorescent intensity than the type and/or amount of fluorophores in the skeleton.

The effect on the colour of fluorescence: As shown in Figure 2.12, reducing porosity by crushing appears to result in a shift in fluorescent emissions to shorter wavelengths. In other words, artificially changing porosity appears to affect the colour of fluorescence.

Figure 2.12 The effect of decreasing porosity by crushing. From left to right the coral (TS-3-89) was crushed to 150-355 μm, 40-150 μm and less than 40 μm. Top row = dull band material. Bottom row = bright band material. The coral is surrounded by boric acid (white) which prevents the pellet from breaking up.

Although these results suggest that coral fluorescence is directly related to porosity, when bright and dull band pellets, which had been ground to 40 μm, are compared with a pellet made from analar $CaCO_3$ (synthetic calcite supplied by the Aldrich Chemical Company also ground to 40 μm), no significant difference in the colour of fluorescent emissions is detected (see Figure 2.13).

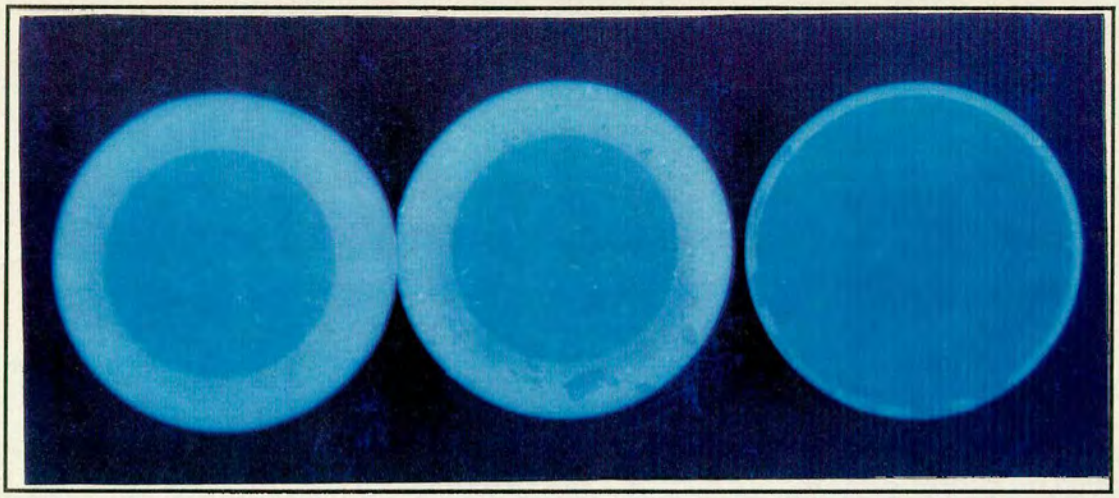


Figure 2.13 Comparing the fluorescent emissions of bright and dull band skeletal material crushed to 40 μm with analar $CaCO_3$ also crushed to 40 μm, when excited with u/v light. From left to right: bright band pellet, dull band pellet (both from TS-3-93) and analar $CaCO_3$.

Although the blue colour seen in the analar $CaCO_3$ pellet could be due to organic impurities, the fact that no significant difference (to the eye) was detected when a pellet was made from analar $CaCO_3$ which had been soaked in NaOCl suggests that this is an unlikely senario (see Figure 2.14). Therefore, an alternative explanation was required to account for the blue fluorescence of the analar $CaCO_3$ pellet.

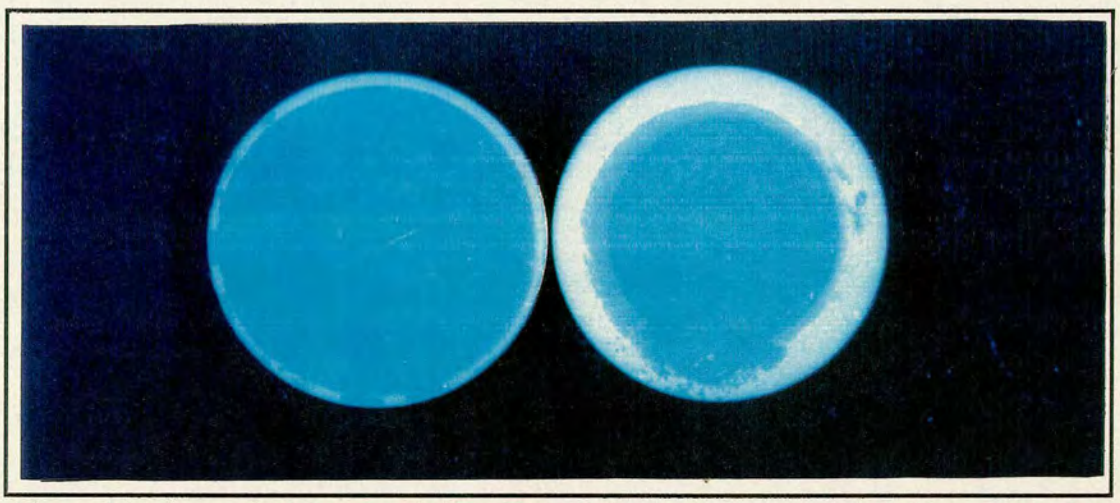


Figure 2.14 Comparison between (left) a 40 μm pellet made from analar $CaCO_3$ powder that was not soaked in NaOCl and (right) a 40 μm analar $CaCO_3$ powder that was soaked in NaOCl before being made into a pellet.

An emission spectrum of the u/v lamp used to excite all coral samples for the purposes of photography and visual observations show that although emissions are centred about 370 nm, enough light at longer wavelengths is emitted to give the lamp

a purple colour (i.e. some light in the visible part of the spectrum is emitted, see Figure 2.15).

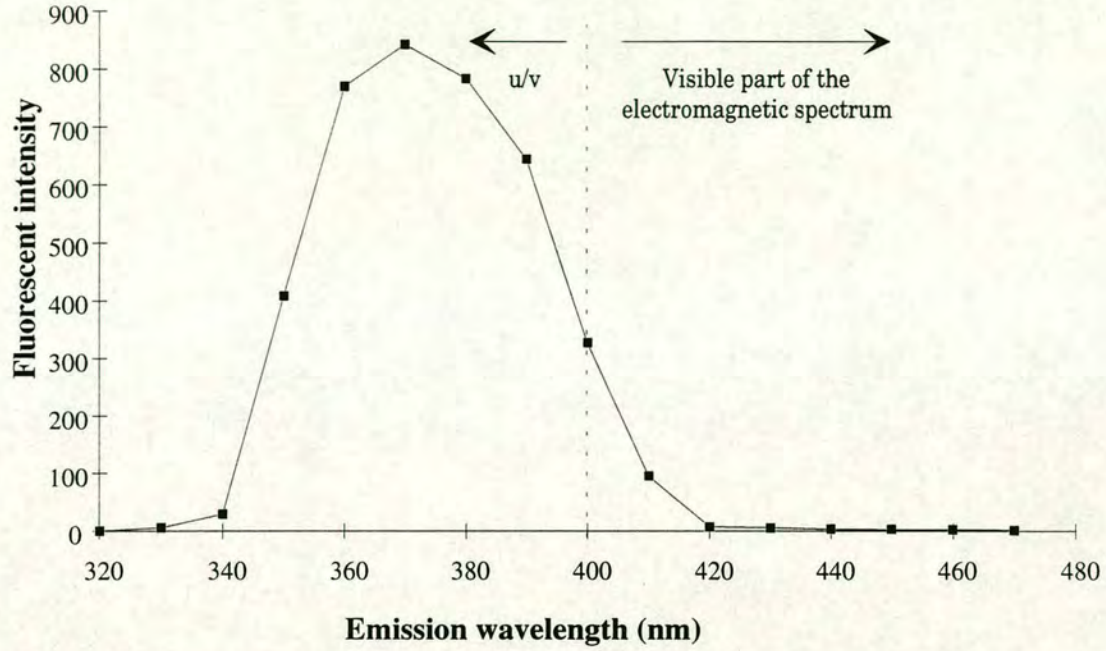


Figure 2.15 u/v lamp emission spectrum.

White objects are generally very reflective and thus appear to be purple when examined under a u/v lamp. As *Porites lutea* skeleton and analar $CaCO_3$ are also white, the majority of the colour seen (purple) when the 40 μm pellets are examined under a u/v lamp is probably due to reflected light and not fluorescence. In order to prevent u/v light reflecting off the coral surface and being recorded on film, a u/v cutoff filter was used. Although it was hoped that only fluorescent emissions would be recorded on film, the fact that the 40 μm pellets (coral and analar $CaCO_3$) are both blue in Figure 2.14 on page 41 is probably because the u/v cut-off filter was unable to cut out all the reflected/scattered light from the lamp (some of which is in the visible part of the spectrum). When a 360 nm bandpass filter, which only allows u/v light between 355 nm and 365 nm through, was used, no fluorescence was seen from the analar $CaCO_3$ pellet. In other words, the analar $CaCO_3$ pellet is either non-fluorescent or very weakly fluorescent (too weak to be detected by the eye). This therefore provides further evidence to support the hypothesis that the blue colour seen from the analar $CaCO_3$ pellet was due to reflected/scattered light from the lamp that was not cut out by the u/v cut-off filter. When coral pellets in which the coral had been crushed to 40 μm were also examined in this way, some fluorescence was seen. However, it was not possible to differentiate between bright and dull bands as

only a very small amount of fluorescence was emitted. As fluorescent banding is seen from uncrushed solid coral surfaces, fluorescence must exceed reflection. This also must to be true of coral skeleton ground to between 150-355 μm as bright and dull bands could still be differentiated (see Figure 2.12 on page 40). When coral was ground to less than 40 μm it was no longer possible to differentiate between bright and dull bands which could be because reflection exceeds fluorescence. Thus, the apparent shift to shorter wavelengths when porosity is reduced by crushing could be due to a more reflective surface rather than to a real change in the colour of fluorescent emissions.

This explanation does not, however, explain why pore spaces appear to a lighter shade of blue (i.e. a different colour) than non-pore spaces (see Figure 2.1 on page 24). One possible explanation that could account for this is that in the pore spaces, a certain amount of the fluorescent emissions are absorbed by the skeleton and then re-emitted at longer wavelengths. As emission always occurs at a longer wavelength than absorption, if blue fluorescence is absorbed by the coral skeleton, the resulting fluorescence is likely to be green or yellow. If this happens to a significant degree, then the light that eventually reaches the eye is likely to be of a longer wavelength than that which would have been emitted initially if it were not re-absorbed.

While pore spaces may be able to affect the colour of fluorescent emissions, the fact that pore spaces are not a significantly different colour suggests that its effects are likely to be minor.

2.5 CONCLUSIONS:

(A) From photographic evidence and the direct measurement of bulk density and porosity, it would appear that no direct link exists between these factors and fluorescent banding.

(B) It would appear that porosity (and therefore bulk density) can affect the intensity of coral fluorescence. As results suggest that coral fluorescence is a surface phenomenon, the greater the surface area exposed to u/v light, the greater the intensity of fluorescent emissions. Conversely, the lower the surface area, the weaker the fluorescence. However, observations made from several different species of coral suggest that porosity (and therefore bulk density) have less of an effect on

fluorescent intensity than the type and/or concentration of fluorophores/g of coral skeleton.

(C) As the colour of most objects appears to change as the amount of the light emitted increases (i.e. it appears to become lighter), it is very difficult to determine whether the difference seen between pore spaces and non-pore spaces is one of colour, intensity or both. Bearing this in mind, results from this chapter suggest that increasing porosity could result in a shift towards longer wavelength emissions as a consequence of the re-adsorption of fluorescence and its subsequent emission at longer wavelengths.

CHAPTER 3. MICROPOROSITY

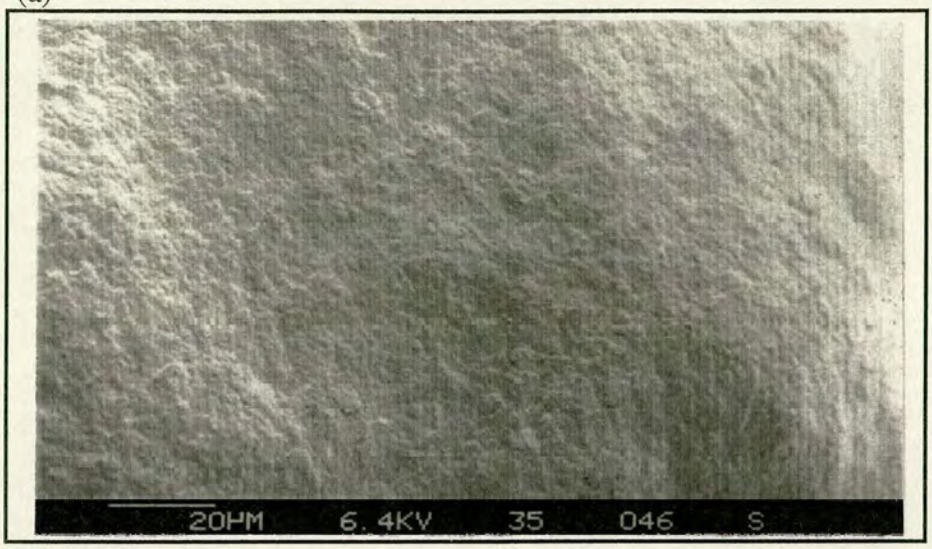
CHAPTER 3. MICROPOROSITY

3.1 INTRODUCTION:

Results presented in Chapter 2 suggest that porosity could affect the intensity, and possibly even the colour of fluorescence in *Porites lutea* from Ko Phuket, Thailand. However, with pore spaces ranging in size from 300 μm to 1.5 mm, the effects of microporosity were largely ignored. Although microporosity can occur as a result of a number of different processes (e.g. microboring activity and crystal growth), work in this chapter concentrates on the effects of microboring organisms. Scanning electron microscopy (SEM) has shown that microborings are not generally found at the living coral surface and only really occur below the green algal band in those specimens of *Porites lutea* that were alive at the time of collection (see Figure 3.1). This therefore suggests that the green algal band is a main site of microboring activity in these corals. The concentric (ring) nature of both the fluorescent bands and the green algal band could suggest a possible relationship between the two. Thus, work in this chapter has concentrated on the effects of algal microborers on coral fluorescence.

Although microboring activity may increase porosity in the short term, its ability to do so in the long term depends, to a greater extent, on the amount of cementation that occurs. In shallow warm seas supersaturated with $CaCO_3$, vacated borings may be filled with acicular aragonite and/or rhombohedral Mg-calcite. However, no signs of carbonate precipitation were found in coral cores from Ko Phuket. Thus, it would appear that conditions are not ideal for carbonate cement precipitation in this area. Scoffin (*pers. comm.*) suggested that this is probably because high levels of rainfall runoff reduce salinities sufficiently to prevent the precipitation of calcareous cements even though the waters are supersaturated with respect to the major carbonates. Only minor amounts of cementation were noted by Scoffin and Tudhope (*pers. comm.*) in inshore *Porites lutea* from the Great Barrier Reef, Papua New Guinea and Indonesia. Thus, in areas where fluorescent banding has been extensively studied, it would appear that microporosity porosity is not significantly affected by cementation.

(a)



(b)

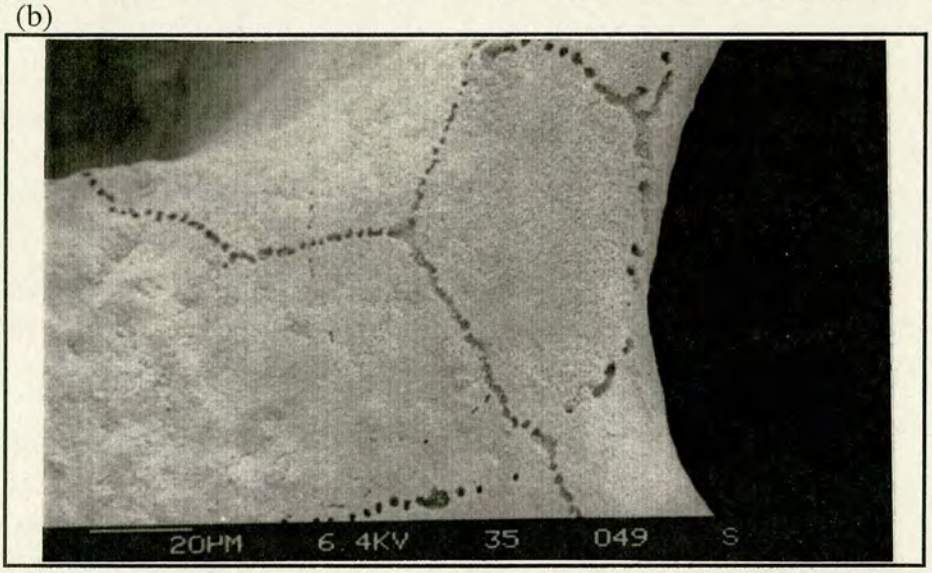


Figure 3.1 Algal microboring activity (a) above the *Ostreobium* band and (b) below the *Ostreobium* band.

The appearance of bright bands in some corals at the living coral surface (see Chapter 1, Figure 1.5a, b, page 10) and dull bands in others (see Chapter 1, Figure 1.15, page 21) would suggest that some form of fluorescent banding may have formed prior to microboring activity. However, it is not known for certain to what extent polyp tissue affects coral fluorescence in this region of the skeleton as the cleaning process is not thought to be completely effective (Gaffey and Bronnimann 1993).

Thus, the aim of this chapter is to asses the role of microboring activity (i.e. microborings less than 100 μm) in the formation of fluorescent bands in corals of the species *Porites lutea* from Ko Phuket that were alive at the time of collection.

3.1.1 Background: The term boring refers to penetration into hard substances by chemical and/or mechanical means while burrowing refers to activities in loose or soft substrates (Bromley 1970). The term endolith has been used to describe organisms that bore into hard substrates. According to DeSalvo (1969), four major groups of microscopically active organisms have been implicated in the penetration and destruction of reef structures. These include sponges, both clinoid and non-clinoid (Goreau and Hartman 1963), a diverse assemblage of boring algae (Purdy and Kornicker 1958), fungi, and bacteria (Purdy 1963). Microboring algae and fungi, which bore via chemical dissolution of the substrate, are abundant in carbonate rocks and sediments in temperate and tropical marine settings. As far as the diameter of the borings are concerned, Golubic *et al* (1975) suggested that they range in size from less than 1 μm to about 100 μm in diameter. In their upper size region they overlap with similar structures formed by sponges and bryozoans. A rather more precise definition was provided by Scoffin (1987) who suggested that algae excavate tubes which are up to 15 μm in diameter while fungi excavate tubes which are between 1-4 μm in diameter. The potential for microboring organisms to increase porosity would initially appear to be quite high as experimental work has shown that microborers quickly infest tropical carbonates in water depths less than 30 m. For example Kobluk and Risk (1977) described the total removal of the outermost 30 μm of an experimental carbonate substrate by boring algae in about 213 days while Tudhope and Risk (1985) suggest that up to 350 $g/m^2/yr$ of $CaCO_3$ can be dissolved in carbonate sands. These figures, however, relate to exposed carbonate surfaces and much lower values are expected when the skeleton is covered by living polyp tissue.

The effects of microboring fungi were largely ignored in this chapter for the following reasons:

(a) Microboring fungi commonly form borings which are 1-4 μm in diameter. While some of these could be detected, the majority were not for reasons that are given in section 3.4.1 on page 59.

(b) Although it is not possible to comment on the effect of microboring fungi on coral fluorescence, a possible argument against fungi being a major influence is that as fungi are not light limited, they can presumably infest across bands. Thus, a concentric banding pattern would probably not be expected.

Thus, work in this chapter has concentrated on the effects of microboring algae.

Microboring algae: As algae require light to photosynthesise, their cells are full of chloroplasts and as a consequence of this they are generally green in colour. Endolithic algae are commonly visible as one, but sometimes two green bands just below the living polyp tissue. Although often referred to as the "*Ostreobium* band" after the blue-green alga *Ostreobium*, this feature may be composed of several genera of endoliths (Risk and MacGeachy 1978). Boring algae have been reported from a maximum water depth of 370 m (Lukas 1978) but are most common in water depths less than 20-30 m (Bromley 1970).

It has been suggested that the development of these highly pigmented endolithic green algal bands occurs where and when conditions within the coral skeleton are optimal for vigorous growth. Highsmith (1981) suggested that such conditions may be analogous to phytoplankton blooms where small populations expand rapidly when conditions such as light, seawater temperature and nutrient concentration are optimised. Much debate has centred on whether these endolithic green algal bands remain in the skeleton all year round. For example, Roos (1967) reported the presence of an endolithic green band in *Porites astreoides* from Curaçao from May to September only. Buddemeier *et al* (1974) reported multiple endolithic green bands in *Goniastrea retiformis* from Eniwetok. The fact that the algal bands were 2-2.5 cm apart while the coral's growth rate was measured at approximately 6 mm/year implied that the bands were not annual. Buddemeier *et al* (1974) therefore suggested that boring algae periodically re-infest corals. On the other hand, various authors (Lukas 1973; Kobluk and Risk 1977) have suggested that *Ostreobium* penetrates coral skeleton shortly after the colony establishes itself and moves up through it as the coral grows. Evidence to support this hypothesis was provided by Highsmith (1981) who reported a yellow-green stain from the single endolithic band in *Goniastrea retiformis* down to the base of the skeleton. Above the endolithic green band the coral skeleton was white. Although a satisfactory explanation that accounts for the presence of multiple bands has not yet been found, the longer the microboring organisms stay in the skeleton, the more bored the coral skeleton will become.

3.1.2 Layout of this chapter: Work begins with an examination of the endolithic green band in unbleached corals collected during the wet and dry seasons. As these bands are sites of active microboring, the nature in which they bore could suggest ways in which their activities may affect coral fluorescence. Algal microborings are then examined using a fluorescence microscope to see what effect they have on coral fluorescence. Finally, the abundance of algal microborings with a diameter greater

than 4 μm is determined in bright and dull bands from *Porites lutea* using images generated by an electron microprobe in backscatter mode.

3.2 THE ENDOLITHIC GREEN ALGAL BAND IN *PORITES LUTEA* FROM KO PHUKET:

In the interests of hygiene, corals were bleached before being shipped back to the UK. For this reason, very few unbleached samples were preserved. However, several coral heads and a number of cores were photographed before bleaching and at least one distinct green algal band was seen in every species of coral sampled with the possible exception of *Favia spp.* (see Figure 3.2).

Figure 3.2 Endolithic green algae in a variety of coral species. From left to right and top to bottom, *Porites lutea*, *Favia spp. (x2)*, *Coeloseris*, *Goniastrea retiformis*, *Platygyra spp.* (x2) and *Goniastrea aspera*. These corals were collected from Tin Smelter Bay in July 1993 during the wet season. The coin is 18 mm in diameter.

In *Porites lutea*, endolithic algae were commonly visible as one, but very occasionally two, green bands just below the polyp tissue layer (see Figure 3.3).

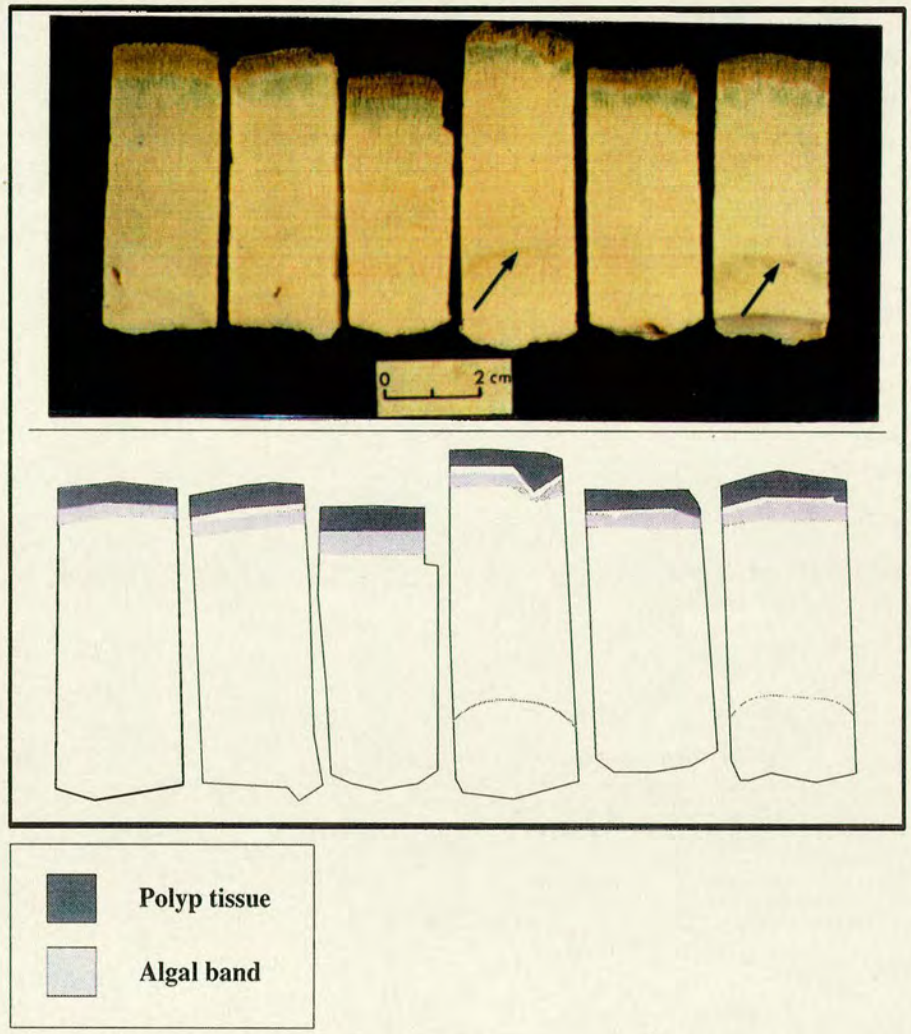


Figure 3.3 Endolithic green algae below the polyp tissue layer in *Porites lutea*. These corals were collected from Tin Smelter Bay in July 1993 during the wet season. The schematic diagram beneath the photograph shows the position of the main algal band and the second (yellow-green) algal band in two of the samples. Arrows indicate the position of the second (green-yellow) algal band in the photograph.

As both field trips were conducted during the wet season, dry season unbleached *Porites lutea* (collected in January 1990) were provided by Scoffin and Tudhope. Bearing in mind the difference in age between the two sample sets, no significant difference was detected in the thickness and/or colour of the endolithic green bands. However, without a larger data set, it was not possible to make a statistically meaningful comparison between the seasons. The fact that the endolithic green bands were found during both the wet and dry seasons could be used to suggest that algal bands remain in the skeleton all year round thus increasing the potential for microboring. However, when two algal bands were seen, the lower band was almost yellow in colour (see Figure 3.4a).

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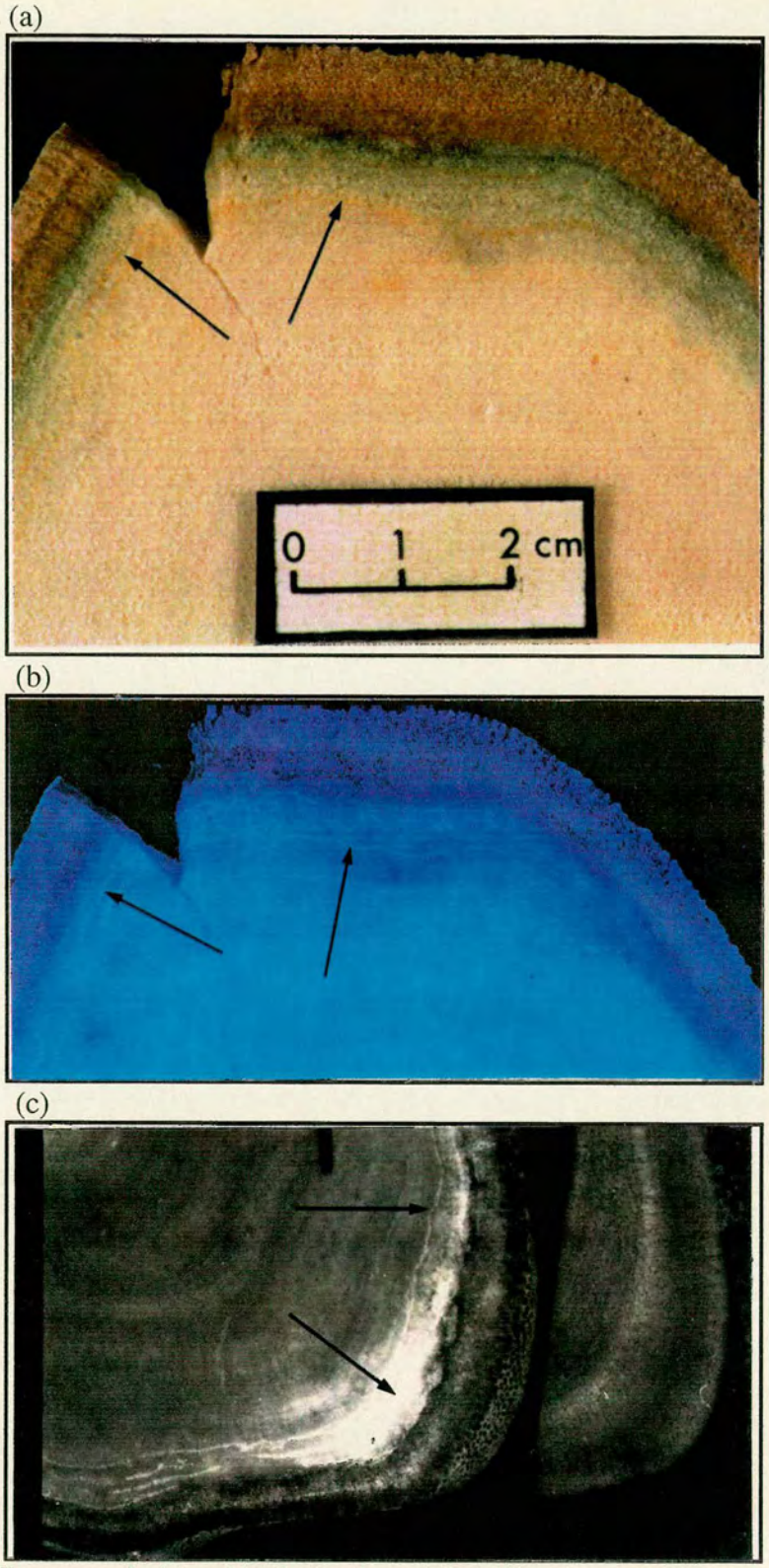

Figure 3.4 The green algal band in *Porites lutea* (PB-1-90): (a) normal light, (b) u/v light before bleaching, (c) u/v light before (left) and after bleaching (right). It can be seen that the yellow-green bands, which are just below the green band (arrows indicate their position), give rise to two highly fluorescent bands. However, evidence suggests that these do not survive the cleaning process as shown in (c).

This yellow band could be a decaying relic of a previous band perhaps providing evidence of multiple invasions. The somewhat conflicting evidence seen in *Porites lutea* from the Thai study area highlights the dilemma already discussed in section 3.1.1. As it is not the aim of this thesis to determine whether microboring algae are present in the skeleton all year round or re-infest periodically, this subject is not discussed any further.

When these green algal bands were examined under a u/v lamp, three things became apparent: (1) they were not fluorescent, (2) they appeared to inhibit coral fluorescence, and (3) some of their breakdown products, which give rise to the yellow-green bands beneath the main green band, were fluorescent (see Figure 3.4a). The ability of algal material and their breakdown products to influence coral fluorescence depends, however, on whether they survive the cleaning process. Although Gaffey and Bronnimann (1993) has shown that the cleaning process is not 100% effective, it would appear that the amount of material left behind was not sufficient to affect coral fluorescence (see Figure 3.4c and Figure 1.12 on page 17 in Chapter 1).

Although Alexandersson (1972) reported a tendency for microborers to avoid emerging from the substrate, when the endolithic green band in *Porites lutea* was examined using a Leica Wild PMS 46/52 binocular microscope, the algal microborers appeared to be at or very near the surface of the skeleton (see Figure 3.5). If this is the case, it would confirm work by Schroeder (1972) who reported that the endolithic green alga *Ostreobium* leaves its boreholes and assumes a chasmolithic habit in larger cavities within the substrate (i.e. adheres to the surface of cavities within the substrate). As these microborings may increase the permeability of the coral skeleton, lattice bound organics may become more susceptible to oxidation which in turn may affect the fluorescent properties of the coral skeleton. In addition to this, the algal microborings may also act as traps for impurities such as clays and silica (the significance of this is discussed in section 3.3 on page 55).

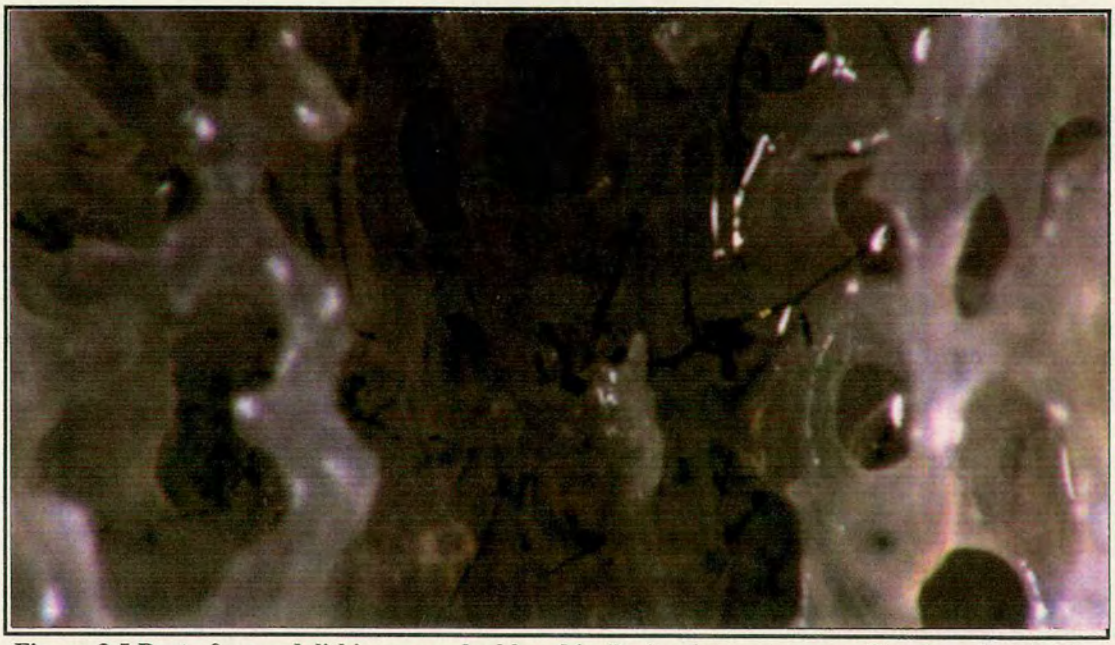

Figure 3.5 Part of an endolithic green algal band in *Porites lutea* at a magnification of x64. The picture is the correct way up as illustrated by the darker green algal boring at the top of the picture and the lighter green-yellow borings at the bottom. Although borings grow in all directions, there appears to be a slight vertical predominance suggesting upward growth probably in response to coral linear extension possibly suggesting an all year round presence. The width of view is ~0.55 mm.

To summarise, although algal breakdown products appear to be fluorescent, their effect on coral fluorescence is likely to be minimal as they are almost completely removed during the cleaning process. This is not to say that the holes they produce do not affect coral fluorescence in some way.

3.3 MICROBORINGS VIEWED UNDER U/V LIGHT:

The aim of this part of the study is to determine whether microborings could affect coral fluorescence. Work in Chapter 2 suggests that increasing porosity results in an increase in the intensity of fluorescent emissions. Thus, one might expect algal microborings to increase fluorescent intensity. However, when microborings are examined using a fluorescence microscope, they appear to be less fluorescent than the surrounding coral skeleton (see Figure 3.6).

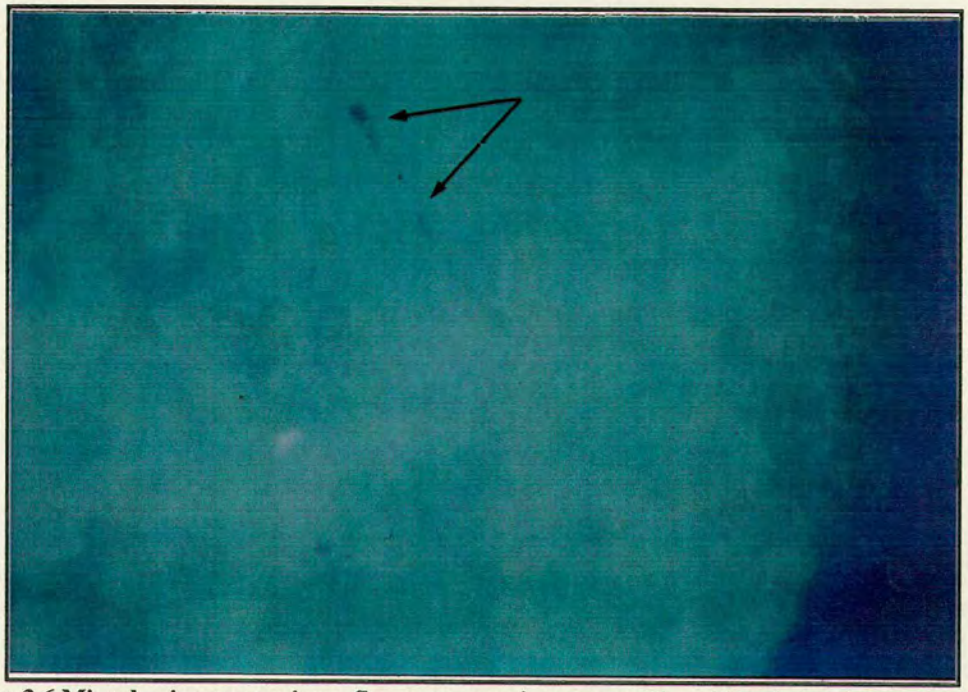

Figure 3.6 Microboring seen using a fluorescence microscope. The width of view is approximately 240 μm. The microboring is indicated with an arrow and is lying in an approximately NW/SE direction.

These observations suggest that microboring activity may actually result in a decrease in fluorescent intensity. Although the reason for this was not absolutely identified, the following possibilities are suggested:

(a) Heavily bored parts of the coral skeleton are generally brown in colour and not very fluorescent (see Chapter 1, Figure 1.11, page 16). In order to determine what causes this, these brown discoloured areas of the skeleton were dissolved in 1N HCl to remove the carbonate. The solutions thus produced were evaporated to dryness and run through an X-ray diffractometer (XRD). Results showed that these areas contained large amounts of the clays kaolinite, illite as well as muscovite and silica. Although these impurities are not found in areas free from the visible signs of boring when analysed using the XRD, small amounts were found when these areas were examined using an electron microprobe (see Figure 3.7). Thus, it is possible that the presence of clays, which gain access to the skeleton as a consequence of macro and microboring activity, are the reason for the reduction in coral fluorescence (the effect that silicates have on coral fluorescence is discussed in Chapter 4, section 4.8.3, pages 145-148, 'Humic acid complex formation'. However, if clays are to reduce the fluorescent intensity of algal microborings, then they should be

associated with the borings. This, however, did not appear to be the case as shown in Figure 3.7 in which clays were not seen in the borings. Thus, while clays may have the ability to reduce coral fluorescence, they may not be the only factor that influences the fluorescence of algal microborings.

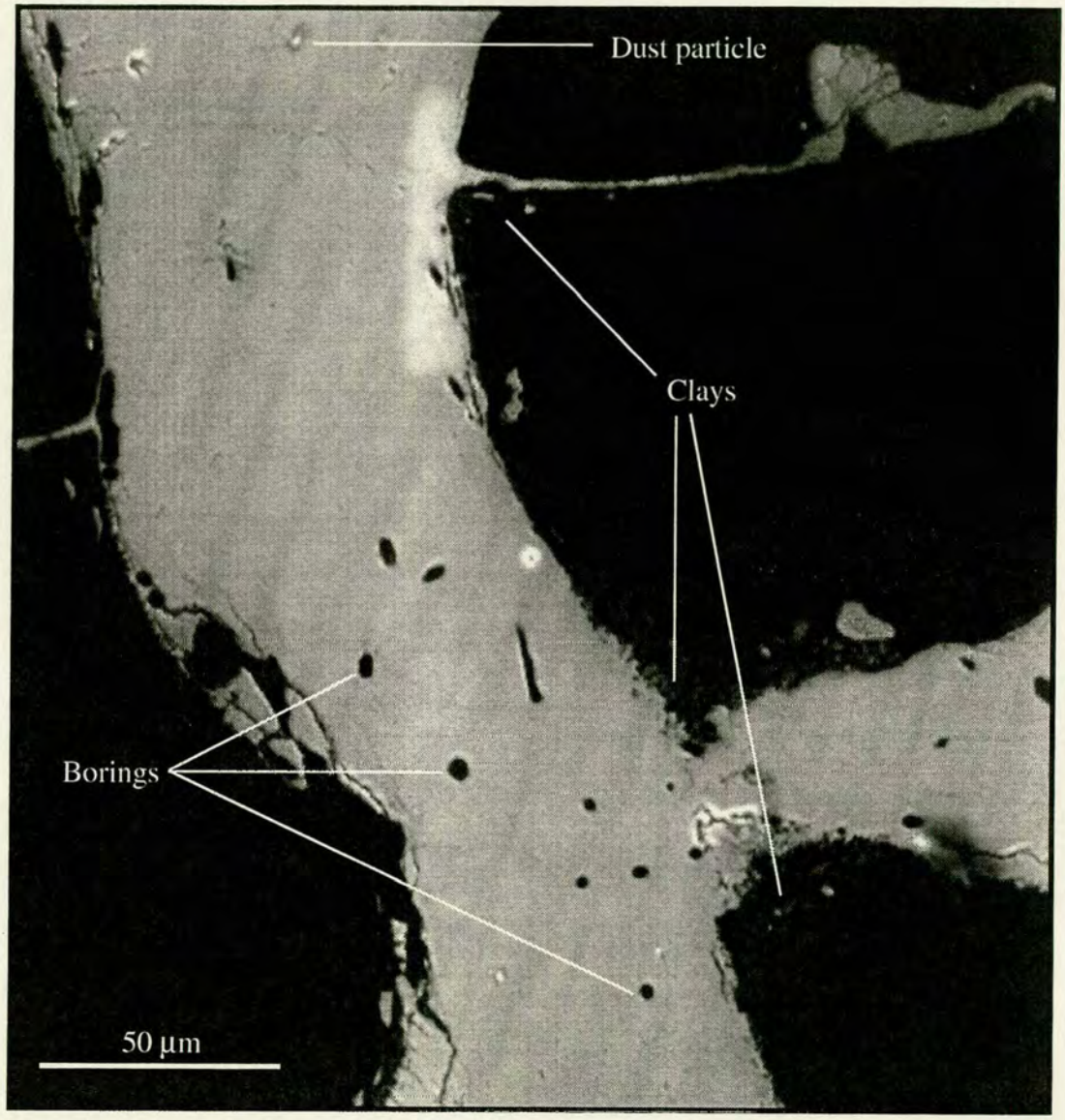

Figure 3.7 Backscattered electron image of a coral skeleton. Except where indicated, the light grey area represents coral skeleton and the large black areas are pore spaces. Examples of microborings, clays and dust particles are indicated. It can be seen that the clays overlay the coral, especially in the crevices.

(b) Although u/v light may be able to enter a microboring, the fluorescent emissions that are produced may be reabsorbed by the skeleton. One possible outcome of this is that very little fluorescence emerges from the microboring. This is demonstrated in Figure 3.8 which shows that when a number of holes were drilled into the surface of *Porites lutea*, the smaller holes (with a diameter of 1 mm and 3 mm) did not appear to fluoresce when they were excited (by u/v light) and viewed from directly above. The rim region of the hole does, however, fluoresce, but it is only seen when viewed at a very acute angle.

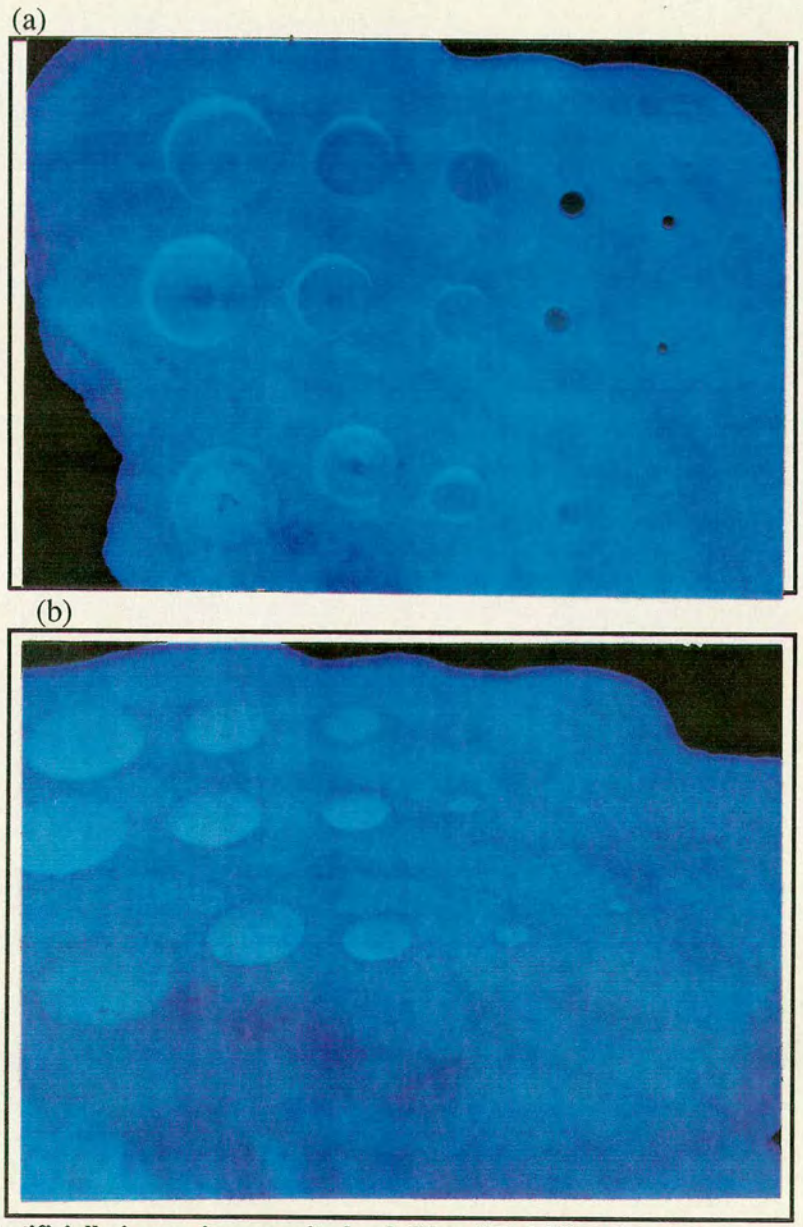

Figure 3.8 Artificially increasing porosity by drilling (SB-6-88). The u/v lamp was perpendicular to the coral surface in both cases. The diameter of the holes from left to right is 12 mm, 10 mm, 6 mm, 3 mm, and 1 mm. The depth of the holes from top to bottom is 10 mm, 5 mm and 2.5 mm. (a) viewed perpendicular to coral surface, (b) viewed at an acute angle.

(c) Fluorescent emissions could be quenched by a non-fluorescent coating secreted to the walls of the borings by the microboring organisms. Bishop (1988) reported the precipitation of barite (barium sulphate) in micro-environments containing decaying organic matter. He suggested that decaying algae and fungi release sulphur which combines with barium to form barite which precipitates on the sides of the microborings. However, Allison (1994) has reported that while some boreholes in *Porites lutea* from Ko Phuket contained a smooth amorphous coating, most boreholes were not diagenetically altered.

To summarise, it would appear that the presence of clay minerals in the microborings and/or some ill-defined small pore effect are the most likely causes of the reduction in fluorescent intensity. Thus, microboring organisms could conceivably cause fluorescent banding if one band contained more microborings than the other (i.e. dull bands were formed during periods of increased microboring activity).

3.4 THE NUMBER OF MICROBORINGS IN BRIGHT AND DULL BANDS:

The aim of this part of the study is to determine whether there is a significant difference in the number of microborings in bright and dull bands.

3.4.1 Methods and materials: The number of microborings in bright and dull bands was determined using an electron microprobe in back-scatter mode. Reasons for its choice will be given later. To be analysed in this way, thin sections were made from coral skeleton that fulfilled the following criteria.

(a) The coral skeleton had to be overlain by living polyp tissue at the time of collection as work by Scoffin (unpublished) has shown that extensive microboring occurs in areas not overlain by living polyp tissue. If this area was sampled, algal microboring activity would be overestimated.

(b) Thin sections were made from coral skeleton below the *Ostreobium* band as skeleton overlain by living polyp tissue at the time of collection contains virtually no microborings above the *Ostreobium* band (see Figure 3.1 on page 47). If this area was sampled, algal microboring activity would be underestimated.

Before collecting a large amount of data, it was considered sensible to determine the following: (1) whether there were sufficient microborings to affect coral fluorescence, and (2) whether there was a significant difference in the number of microborings between bright and dull bands. Thus, thin sections were made from adjacent bright and dull bands from a single colony of *Porites lutea* (TS-10-88). Both longitudinal and transverse sections were taken. All thin sections were impregnated with epoxy resin (araldite) to provide structural support before being polished.

The microprobe works by firing a beam of electrons onto the surface of the specimen. Electrons back scatter off the sample and the number that do so is proportional to the atomic number of the various components in the sample. The darker the area, the lower the mean atomic number. Although the microprobe can generate images with a magnification of x80,000, the amount of area covered by one back-scattered electron image would be so small that a considerable number of back-scattered electron images would be required before a representative sample was obtained. As algae are believed to be the dominant microflora in these corals, and as Tudhope and Risk (1985) suggested that fungal borings are volumetrically insignificant in shallow water carbonate sediments, a magnification of x300 was used as this allowed microborings with a diameter of 4 μm to be reliably observed and meant that fewer images were required. Although smaller borings could be seen at this magnification, it was not always possible to distinguish them from other surface features such as dust. Thus, by only measuring borings greater than 4 μm, most of the fungal microborings were probably missed out. Although the backscatter images were 17 cm x 17 cm, at a magnification of x300, this effectively represents an area that is 3.211×10^{-3} cm^2 (i.e. $5.67 \times 10^{-2} \times 5.67 \times 10^{-2}$). Readings were taken every cm (or 1.89×10^{-4} cm at a magnification of x300) as no significant difference was detected in the level of microboring activity when readings were taken every 0.5 cm (or 9.44×10^{-5} cm at a magnification of x300), i.e. no significant difference was detected when the number of counts was increased from 289 (17 x 17) to 1225 (35 x 35) (see Appendix A3.1). By taking 20 images from each thin section, a total area of 0.064 cm^2 was covered. Although it is acknowledged that this is not a very large sample area, around 300 back-scattered electron images at this magnification would be needed to cover an area of 1 cm^2. A reflected light microscope was the obvious alternative and although microborings could be easily identified, the field of view was considerably smaller than the electron probe's at similar magnifications. Thus, an even greater number of images would have to be taken to cover the same area.

Microborings are also commonly studied using multipurpose embedding techniques (full details given in Golubic *et al* 1970). However, impregnating the borings and then dissolving away the carbonate would make it difficult to calculate the amount of $CaCO_3$ that was bored, which is why this was not done. Figure 3.7 is a typical backscatter image from which measurements were obtained.

In order to determine the errors associated with the point counting method, one particular image (TS-10-88 DB1) was counted eight times. Results (see Appendix A3.2) showed that the error (standard deviation as a percentage of the mean) associated with the estimate of borings as a percentage of skeletal matter was 4.65%.

3.4.2 Results: Although results in Table 3.1 show that dull bands have more microborings than bright bands, a Mann-Whitney U-Test failed to detect a significant difference between the two data sets.

Table 3.1 Microboring activity in bright and dull bands (TS-8-88).

Sample	% Skeletal matter	Borings as a % of skeletal matter	Sample	% Skeletal matter	Borings as a % of skeletal matter
BB1	47.30	1.02	DB1	37.01	2.01
BB2	48.37	1.00	DB2	30.52	1.30
BB3	38.75	1.32	DB3	38.58	1.17
BB4	39.45	0.88	DB4	37.87	2.10
BB5	35.31	1.52	DB5	39.53	1.01
Mean	**41.84**	**1.15**	Mean	**36.70**	**1.52**

Mann-Whitney U-Test:

Sample	Borings as a % of skeletal matter	Rank	Sample	Borings as a % of skeletal matter	Rank
BB1	1.02	4	DB1	2.01	9
BB2	1.00	2	DB2	1.30	5
BB3	1.32	6	DB3	1.17	7
BB4	0.88	1	DB4	2.10	10
BB5	1.52	8	DB5	1.01	3
Sum		21	Sum		34

U1	19
U2	6

Critical values for the lowest U value are given below:

99%	0
95%	2
90%	4

This suggests that microboring activity is not the main control on fluorescent banding, as a significant difference in the amount of microborings in bright and dull bands would be expected if it was. In addition, the level of microboring in TS-8-88, which was calculated to be between 1 and 2% (in agreement with Lukas (1974) and Highsmith (1981)), would appear to be too low to have a significant effect on coral fluorescence. As no significant difference in algal microboring activity was detected between bright and dull bands in TS-8-88, no further point counting was performed.

To summarise, these results suggest that algal microboring activity is not the main cause of fluorescent banding and although microborings do appear to reduce fluorescence in *Porites lutea* from Ko Phuket, the small number of microborings suggests that the effects on fluorescent intensity are only minor.

3.5 CONCLUSIONS:

(A) Evidence suggests that microboring algae are present in the coral skeleton at all times of the year thus increasing the potential for microborings.

(B) When examined under a u/v lamp and using a fluorescence microscope, algal microborings can be seen to reduce coral fluorescence. Although it is not certain exactly how this occurs, the most likely explanation is that the microborings trap particles of clay and/or prevent the fluorescent emissions from leaving the borehole as a consequence of reabsorption.

(C) Although there were more algal microborings in dull bands than bright bands, the difference was not significant even at the 90% confidence level. As a significant difference in the number of microborings between bright and dull would be anticipated if algal microboring activity was to cause fluorescent banding, it is concluded that microborings are not the main cause of fluorescent banding. Evidence would also suggest that the number of algal microborings is not sufficiently high to have a significant effect on coral fluorescent intensity.

CHAPTER 4. THE FLUORESCENCE OF CORAL SOLUTIONS

CHAPTER 4. THE FLUORESCENCE OF CORAL SOLUTIONS

4.1 INTRODUCTION:

Bright and dull band emission spectra were originally produced by Boto and Isdale (1985) who excited coral skeletons of the species *Porites lutea* with longwave u/v light (360 nm) and measured emissions between 400 nm and 700 nm. The resultant emission spectra had one broad featureless peak with a maxima at 440-460 nm which they attributed to humic acid fluorescence (see Chapter 1, Figure 1.3, page 6). However, work by Coble *et al* (1990) and Matthews *et al* (in review) has shown that a greater amount of information can be obtained from the production of excitation/emission (EXEM) spectra. These are obtained by combining a number of individual excitation spectra taken over a range of emission wavelengths, details of which are discussed in section 4.3.2 (see page 72). For a full review of previous work see Chapter 1, section 1.2, pages 2-9.

4.1.1 Objectives: The principal aim of this chapter is to investigate the fluorescent characteristics of coral fluorophores (both humic and non-humic) from bright and dull bands. In order to achieve this, the coral skeleton was dissolved using dilute HCl which: (1) released the fluorophores thus allowing them to be studied more effectively, and (2) eliminated the effects of porosity which have been shown to affect fluorescence (see Chapter 2 for the effect pore spaces have on coral fluorescence). Using industrial humic acid (for reasons given later), work in this chapter also attempts to determine the effects of the following on humic acid and coral fluorescence: (1) fluorophore concentration, and (2) the presence of metal ions such as Ca^{2+}, Fe^{3+}, Mn^{2+}. Finally, various soil, sediment and seawater samples were examined in an attempt to identify the source or sources of coral fluorophores.

4.1.2 Layout of this chapter: Since there are many different factors which may affect the fluorescence of coral solutions as well as a variety of techniques for assessing the importance of each of these factors, this chapter has been subdivided into a number of sections. Each section is a self-contained entity in that it starts with a brief introduction describing the rationale behind the piece of work, continues with a description of the materials and methods and ends with the presentation and interpretation of the results. The final section in this chapter pulls together evidence

from all the constituent sections and presents a summary of the principal conclusions of this aspect of the project.

4.2 SAMPLE PREPARATION:

4.2.1 Dissolution of the skeleton: In order to release the fluorophores, the coral skeleton must first be dissolved. However, before this can be done, suitable bright and dull band samples had to be chosen and prepared (see Chapter 1, section 1.5, pages 15-21). Once this had been done, the coral was crushed to less than 40 μm in an attempt to homogenise the distribution of organics within each bright and dull band sample. Crushing was carried out by hand using an agate pestle and mortar as comparative tests using identical sample masses from a common source have shown that mechanical crushing in a tungsten tema resulted in a decrease in fluorescent intensity (see Appendix A4.1). It was thought that the high temperatures reached in the tungsten tema probably destroyed some of the fluorophores. Dissolution was carried out with HCl according to the following reaction:

$$CaCO_3 + 2HCl \rightarrow CaCl_2 + CO_2 + H_2O$$

Using this formula, the amount of HCl required to dissolve a certain mass of coral skeleton can be determined. In order to test whether the concentration of HCl used to dissolve the coral skeleton affected the fluorescent properties of the coral fluorophores, five 2 g samples from a common source crushed to less than 40 μm were dissolved using the following concentrations of HCl: 0.4N, 0.8N, 1N, 2N, 2.6N[1]. The solutions were evaporated down to 20 mls in an oven at 50°C and then adjusted to pH 9 (the reasons for evaporating to 20 mls and adjusting to pH 9 are discussed on page 67 in sections 4.2.2 and 4.2.3 respectively). EXEM spectra for all five solutions were essentially the same which suggested that the fluorophores were resistant to acid attack. However, as the risk of protein denaturisation increases with acidity (Mantoura and Riley 1975), coral skeleton was dissolved using 1N HCl. Although a weaker acid solution could have been used, the rate of carbonate dissolution decreased unacceptably. The solutions produced in this way contained Ca^{2+} and Cl^- ions. The presence of calcium ions is of particular importance as Wiley (1974) has suggested that they can enhance the fluorescence of natural waters at low

[1]Normality is the same as molarity when used in conjunction with a monobasic acid such as HCl (i.e. one hydrogen ion per acid molecule) but twice the molarity when it is a dibasic acid (two ions per acid molecule).

pH's. However, as all dissolved solutions were adjusted to pH 9, Ca^{2+} was unlikely to cause a problem. In order to test whether Ca^{2+}, and for that matter Cl^- ions, affected coral fluorescence, 5 g of coral skeleton was dissolved using 1N HCl, concentrated to 20 mls and adjusted to pH 9. This solution was then divided into two equal parts. 2.75 g of $CaCl_2$ (from the Aldrich Chemical company) was then added to one of the parts. In 10 mls of this coral solution there would be 2.5 g of coral and therefore 2.75 g of $CaCl_2$ could in theory form. Thus, by adding 2.75 g of $CaCl_2$, the amount of Ca^{2+} and Cl^- ions is increased by 100%. EXEM spectra show that the addition of $CaCl_2$ has no significant effect on the fluorescent properties of coral EXEM spectra (see Figure 4.1). This is of particular importance as it suggests that coral solutions do not have to have Ca^{2+} and Cl^- ions removed to enable their fluorescent properties to be studied effectively.

Although the presence of Ca^{2+} and Cl^- ions does not appear to affect the fluorescent properties of coral solutions, the crystallisation of $CaCl_2$ resulted in a reduction in fluorescent intensity. These crystals formed when: (1) coral solutions containing excess acid (added to speed up the dissolution process) were evaporated down to 20 mls, and (2) coral solutions containing more than 13.5g of $CaCO_3$ were evaporated down to 20 mls (see Appendix A4.2). $CaCl_2$ crystals which formed in this way were dissolved in 20 mls of distilled water (DH_2O) and adjusted to pH 9 before EXEM spectra were produced. Results suggested that the crystallisation of $CaCl_2$ removed coral fluorophores from solution. The amount of organic material incorporated into the crystals depended on: (1) the amount of organic material in solution, and (2) the amount of crystals that formed. In order to avoid these problems, coral skeleton was dissolved using the minimum amount of HCl.

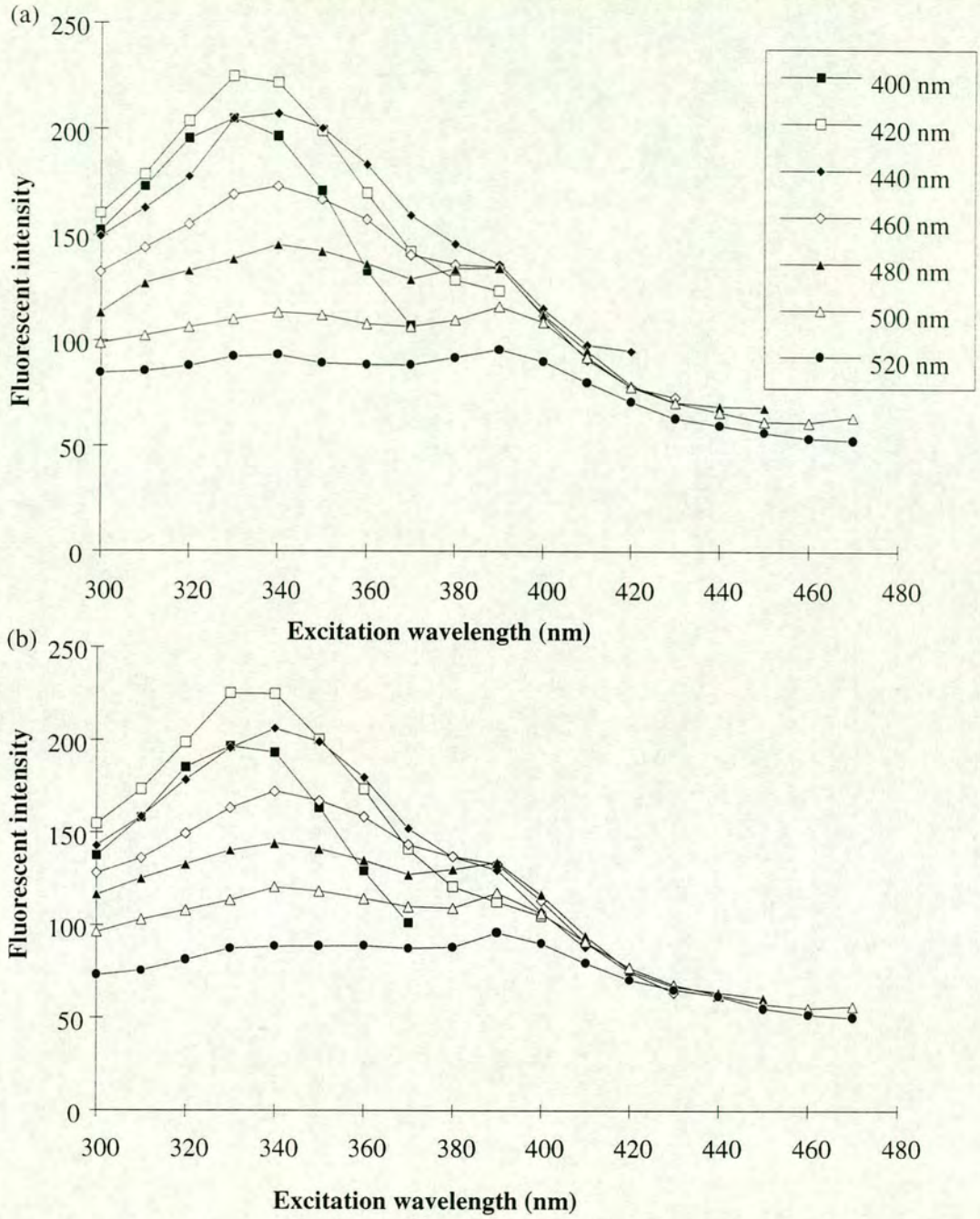

Figure 4.1 The effect of Ca^{2+} and Cl^- ions on coral fluorescence. (a) Coral blank (b) coral blank with 2.75 g of $CaCl_2$. In all diagrams of this nature in this thesis (except where stated), the values presented in the box represent emission wavelengths.

Whenever coral solutions were prepared, the glassware used had to be cleaned effectively. This was because EXEM spectra reproducibility was low when the glassware was only acid washed and DH_2O rinsed. This suggested that some fluorescent molecules had the ability to stick to the sides of the glassware. When the glassware was acid washed, any alkali soluble fluorophores which were stuck to the

sides of the glassware would probably not be removed. Even soaking in DH_2O (approximately pH 7) would only remove the water-soluble fluorophores. Thus, an alkali wash was required to remove the alkali-soluble organics. For this reason, a Meile dishwasher was used to clean all the glassware used in this chapter. The wash cycle consists of an initial alkaline wash followed by an acid wash and then a final rinse using DH_2O. The glassware was then left to soak in DH_2O until required.

4.2.2 Adjusting the concentration of coral solutions: Although 40 mls of 1N HCl are required to dissolve 2 g of coral skeleton, the solutions produced in this way are very weakly fluorescent with little or no fluorescence being recorded at emissions above 500 nm. This problem was exaggerated when XAD-2 resin was used to extract humic acids as very large volumes of eluent were required to desorb the humic acids from the resin (XAD-2 resin use is discussed later in section 4.7, pages 123-132). Therefore, in order to increase the concentration of fluorophores in the solution phase, all samples were evaporated to 20 mls in an oven at 50°C (unless otherwise stated). Obviously the concentration of the final solution will depend on the amount of coral initially dissolved. For example, when 2 g of skeletal material is dissolved and evaporated to 20 mls, a 1M coral solution is produced whereas 10 g of skeletal material, dissolved and evaporated to 20 mls, would produce a 5M coral solution. It should be pointed out that the molar concentration of a particular coral solution refers to the calcium molarity of that solution. 6.7M represents the highest concentration coral solution that can be formed in this way (i.e. 13.5 g of coral skeleton in 20 mls) without crystals of $CaCl_2$ forming. As their formation results in the removal of fluorophores from solution, the concentration of Ca^{2+} ions was kept below 6.7M. Although XAD-2 resin could be used to remove both Ca^{2+} and Cl^- ions thus allowing the fluorophores to be concentrated to an even greater degree, the numerous problems associated with the use of XAD-2 resin precluded its use (see section 4.7). Care was taken not to evaporate samples to dryness as tests using identical samples have shown that lower fluorescent intensities were produced for both bright and dull bands when this happened (see Appendix A4.4).

4.2.3 Adjusting the pH of coral solutions: It has been known for some time that pH affects the solubility of humic acids (Thurman *et al* 1988). What is not known is whether pH affects the fluorescent properties of coral solutions. In order to test this, six 20 ml aliquots of a 5M coral solution were adjusted to the following pH's; 1, 2, 2.75, 7.8 (pH of Thai seawater), 9 and 11, using HCl and ammonium solution, both of which were essentially non-fluorescent. Results showed that although fluorescent

intensity gradually decreased when the solutions were adjusted from pH 11 to pH 2, the shape of the EXEM spectra remained fairly constant. However, below pH 2, the shape of the EXEM spectra changed and fluorescent intensity decreased quite rapidly (see Figure 4.2).

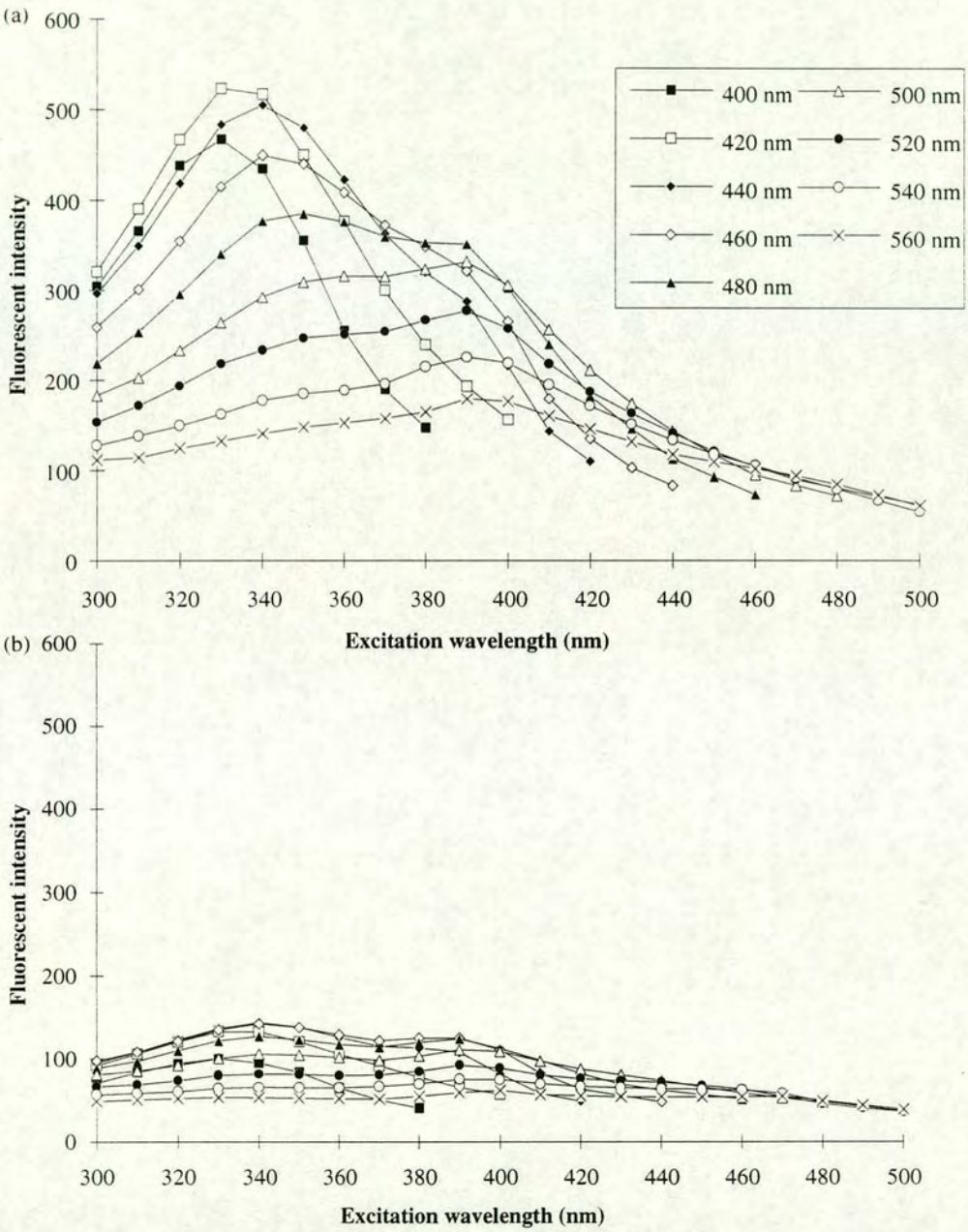

Figure 4.2 The effect that pH has on Aldrich humic acid fluorescence. (a) pH 9, (b) pH 1. The total fluorescent intensity of the pH 1 solution is considerably less than the pH 9 solution.

This change has been attributed to the presence of humic acids which precipitate below pH 2. The possible presence of humic acids in *Porites lutea* from Ko Phuket is discussed in section 4.4.3. To avoid these problems, all solutions were adjusted to

pH 9 once they had been evaporated to 20 mls. This pH was chosen because it was relatively easy to obtain manually and because the EXEM spectra did not vary significantly between pH 7 and 11. Although pH can also be adjusted using buffers, results of several tests suggested that they were unsuitable (see Appendix A4.3).

4.2.4 Do coral fluorophores degrade over time?: It was important to know whether coral fluorophores, once liberated from the skeleton, were prone to oxidative decomposition. If decomposition was rapid, only fresh samples should be compared. In order to determine the stability of coral solution fluorescence through time, the fluorescent intensity of the dominant coral solution excitation peak was measured over 1.5 months. Work in section 4.4.1 has shown that the dominant coral excitation peak occurs when solutions are excited between 320-350 nm and emissions are recorded between 400-440 nm. Thus, the intensity of fluorescent emissions from a 5M coral solution were measured at 400 nm when the coral solution was excited at 335 nm (written as 335/400 nm) and at 420 nm when the coral solution was excited at 345 nm (written as 345/420 nm). A large volume of 5M coral solution was made up so that previously unexcited samples could be analysed on different days. This was important as the aim of this study was to determine the effect of ageing on coral fluorescence rather than the number of times the solution was exposed to u/v light. Each point in Figure 4.3 represents the mean fluorescent intensity of five individual runs taken from one sample after which it was discarded. The solution was kept in a dark cupboard throughout the experiment to prevent photo-degradation. Results in Figure 4.3 show that in general, the fluorescent intensity of both peaks decreased with time. The ups and downs were attributed to instrumental factors (LS-5 instrumental variation is discussed in section 4.3.3).

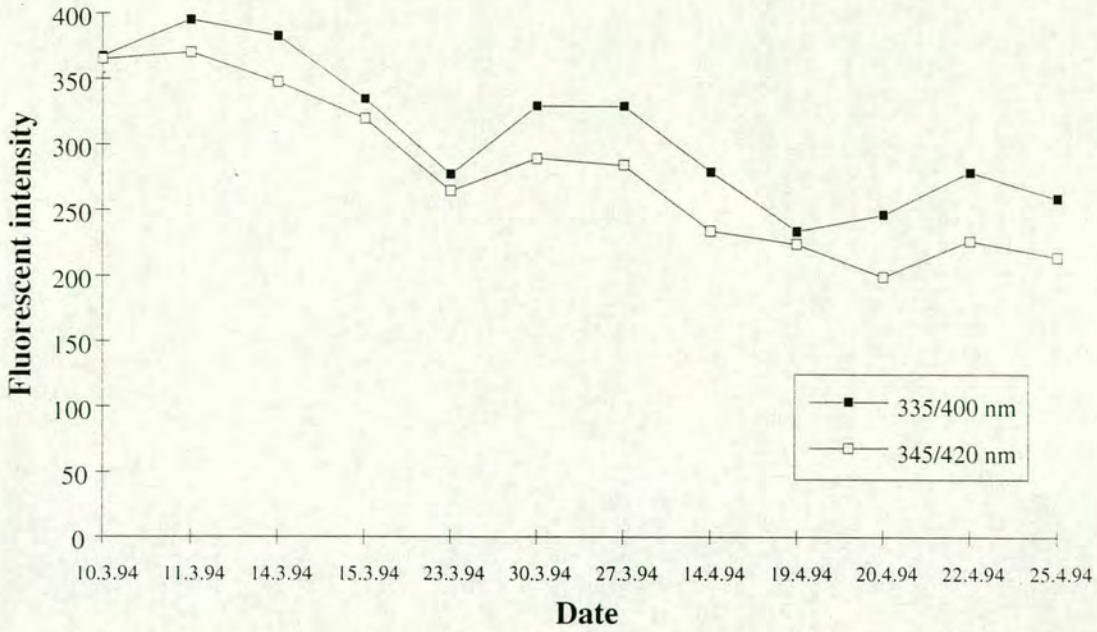

Figure 4.3 The effect time has on the fluorescent intensity of coral solutions. This change has be measured at two excitation/emission combinations: 335/400 nm and 345/420 nm over two months.

Over the 1.5 month period, the intensity of the 335/400 nm excitation/emission peak decreased by approximately 35% whereas the decrease was nearer 45% for 345/420 nm excitation/emission peak. As this represents a substantial decrease in fluorescent intensity, the fluorescent properties of fresh and ageing samples should not be compared. Coral fluorescence has been detected in fossil corals (Klein 1990) which suggests that fluorophore decomposition is lessened by the presence of the skeleton. However, as the decomposition of fluorophores in the solid state was not examined, its effects on coral fluorescence were not ascertained. The fact that coral fluorophores did decompose over a period of 1.5 months could, however, have important implications regarding the use of absolute (as opposed to relative) coral fluorescence as an environmental indicator, either in the solution phase or solid state. This is because, if coral fluorescence is to be used as an environmental indicator, some correction factor would need to be used to allow for ageing of the fluorophores. If this was not done, misleading environmental data would be produced.

4.2.5 Summary/Key points:

(1) Skeletal material was dissolved using the minimum amount of 1N HCl to reduce the risk of protein denaturisation and prevent the formation of $CaCl_2$ crystals.

(2) Because fluorescent intensity was, in general, very weak, coral solutions were concentrated to 20 mls. Care was taken not to evaporate the solutions to dryness as this appeared to affect coral fluorophores in some undefined way.

(3) Because pH appears to be able to affect the shape and intensity of coral solution EXEM spectra, all solutions were adjusted to pH 9 as this was an easy value to obtain manually and kept humic acids in solution.

(4) Once the coral matrix had been dissolved with acid, the coral fluorophores began to decay. For this reason, fresh solutions were used only.

4.3 DATA ACQUISITION AND PROCESSING:

4.3.1 The Perkin Elmer LS-5 spectrofluorimeter: A Perkin Elmer LS-5 spectrofluorimeter and R100A chart recorder were used to obtain EXEM spectra for all coral and humic acid solutions analysed in this chapter. Incident radiation was produced by a special pulsed xenon flash tube. Each flash was of high intensity and short duration and provided a continuum of energy over the spectral range. The precise wavelength used to excite the sample and the wavelengths over which the fluorescent emissions were recorded were determined by the slit widths on the excitation and emission monochromators. Since 5 nm emission and excitation slits were used, the assumption of a monochromatic source was a reasonable approximation for the purposes of colour determination. Fluorescent emissions were recorded by a standard (978 1R) photomultiplier (PMT) which could record emissions between 230 nm and 650 nm. The PMT detector output was processed by ratio-recording electronics and produced a signal (dimensionless) that was proportional to the level of emission from the sample. Although the intensity of the exciting light varied with wavelength, the emission data were corrected to take this into account i.e. emissions assumed equal excitation intensity at all wavelengths. Output emissions, on the other hand, were not automatically adjusted to account for the fact that the PMT does not respond to all wavelengths equally i.e. it is more sensitive to some wavelengths than others. As fluorescent emissions were recorded at 90° to the direction of the excitation beam, transmitted light, which passed straight through the sample, was not recorded. Scattered light was generally not recorded when a gap of 20 nm was left between the excitation and emission wavelengths unless very cloudy or solid samples were analysed. Second order scattering,

produced because a diffraction grating was used to separate the different emission wavelengths, occurs when the excitation wavelength is approximately half the emission wavelength. However, over the range of excitation and emission wavelengths used in this chapter (discussed in section 4.3.2), the problem of second order scattering was generally not encountered. The only exception to this occurred when the fluorescent emissions at 580 nm were recorded, in which case excitation began at 320 nm instead of 300 nm. All solution samples were contained in square section 4 ml cells made of polymethylmethacrylate which is effectively transparent to wavelengths above 270 nm. EXEM spectra were collected at a scan speed of 480 nm/min. Although this was not the optimum scan speed, production time for one spectrum was reduced from approximately one hour to 10 minutes with only a small loss in precision (see Appendix A4.5).

4.3.2 Excitation and emission (EXEM) parameters: EXEM spectra were produced by combining the individual excitation spectra shown in Table 4.1. It should be pointed out that in this thesis, the term 'excitation wavelength' refers to the wavelength of radiation used to excite a particular sample (be it a coral solution or solid coral skeleton).

Table 4.1 Excitation and emission range.

Excitation range (nm)	Emission wavelength (nm)
300-380	400
300-400	420
300-420	440
300-440	460
300-460	480
300-480	500
300-500	520
300-500	540
300-500	560
320-500	580

Before discussing the reasons why the above excitation and emission parameters were chosen, it might be helpful to differentiate between, excitation, emission and EXEM spectra. An excitation spectrum is produced by exciting a sample over a range of different wavelengths and recording the fluorescence emitted at a fixed wavelength. As will become evident throughout this chapter, an excitation spectrum often has peaks along its length, and in this chapter, these peaks are referred to as excitation peaks. An excitation peak occurs when the fluorophores that give rise to

fluorescence absorb energy. The energy absorbed at a particular wavelength is then emitted as fluorescence at a longer (lower energy) wavelength. The excitation peak should not, however, be regarded as an absorption peak for the sample as a whole as not all molecules that can absorb energy at a particular excitation wavelength will emit fluorescence. An emission spectrum is produced by exciting the sample at a fixed wavelength and recording the fluorescent emissions over a range of wavelengths. Although an EXEM spectrum is made up of a number of excitation spectra, it can also be used to produce emission spectra which is why it is so useful.

Although some fluorescence was emitted when coral solutions were excited between 270 nm and 300 nm, excitation peaks were not generally recorded over this range, in addition to which, the fluorescence emitted over this range was often very variable. For these reasons, excitation generally began at 300 nm, although where appropriate, as when examining seawater samples in which a ~275 nm excitation peak can sometimes be seen (see Figure 4.42, page 138), excitation began at 270 nm. 500 nm was chosen as the upper excitation limit because no excitation peaks were detected in coral solutions between 500 nm and 650 nm irrespective of concentration. Although excitation peaks could exist above 500 nm, to be detected they would have to be much larger (more intense) than the 330-345 nm excitation peak as the PMT's response decreases with increasing wavelength. As a difference between bright and dull bands in solid coral skeleton was clearly seen in the visible part of the electromagnetic spectrum, fluorescent emissions were recorded from 400 nm. Fluorescent emissions were recorded up to 580 nm because of the reduction in PMT sensitivity at longer wavelengths than this (see Figure 4.4).

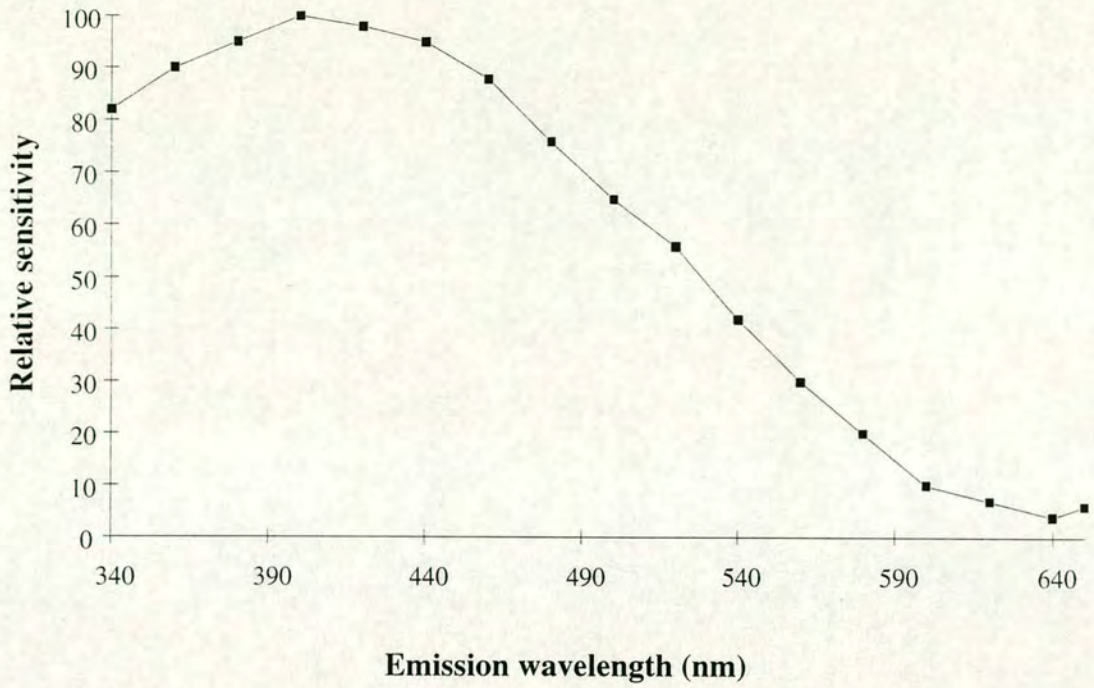

Figure 4.4 Standard (978 1R) PMT response curve. Data supplied by Perkin Elmer.

Once an EXEM spectrum was produced, it was digitised using a Ferranti Free-scanner. The data were then adjusted to account for the variable response of the PMT to different emission wavelengths using the PMT response curve shown in Figure 4.4.

As fluorescent emissions in this chapter are described in terms of both wavelength (i.e. long-wave and short-wave) and energy (i.e. low-energy and high-energy), it is important not to confuse the two. The energy associated with a particular wavelength of radiation is proportional to the square of its frequency. The higher the frequency (and hence the shorter the wavelength) the greater the energy of the radiation. Thus, yellow fluorescent emissions are a longer wavelength, lower energy form of radiation than blue fluorescence.

4.3.3 The errors associated with the LS-5 spectrofluorimeter: The point of this exercise was: (1) to determine the errors associated with an individual EXEM spectrum, and (2) to determine whether the fluorescent intensities of EXEM spectra obtained from different coral solutions could be directly compared. In all cases, the precision (standard deviation as a percentage of the mean) was calculated from a replicate set of six measurements. As u/v and visible light have been shown to affect the fluorescent properties of certain molecules including humic acids (see Kouassi

and Zika 1990; Chapter 6, section 6.3.3, page 186; and Chapter 7, section 7.2.3, page 222), fresh sample was used for each measurement.

The error associated with an individual EXEM spectrum: The error associated with an individual EXEM spectrum was calculated using quinine sulphate, a commonly used fluorescent standard. A fluorescent standard was used because, at any one concentration, each EXEM spectrum should be identical. Thus, any variation recorded in the intensity of fluorescent emissions should be due to the LS-5 spectrofluorimeter. Although quinine sulphate has two excitation peaks (one at 290 nm and the other at 335 nm), the dominant 335 nm excitation peak was used as it was easier to measure (especially at longer emission wavelengths), thus ensuring more accurate results. As the PMT does not record all wavelengths equally (see Figure 4.4), the error associated with an individual EXEM is likely to vary according to emission wavelength. Thus, errors were calculated for all relevant emission wavelengths between 400 nm and 580 nm.

Table 4.2 The errors associated with an individual EXEM spectrum.

Replicate	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm
1	816.00	510.00	290.50	159.80	84.13	44.50	23.05	10.42	4.40	2.40
2	810.00	505.00	288.00	158.00	84.00	43.50	22.90	10.18	4.12	2.04
3	795.00	505.00	286.00	156.40	83.20	43.50	22.40	10.00	4.00	1.88
4	791.00	505.00	285.00	156.20	83.20	43.10	22.40	9.80	3.90	1.76
5	790.00	500.00	285.00	155.20	82.67	42.70	22.05	9.66	3.70	1.64
6	780.00	499.00	280.50	155.00	81.33	41.70	21.95	9.60	3.60	1.56
Mean	797.00	504.00	285.83	156.77	83.09	43.17	22.46	9.94	3.95	1.88
STDEV	13.48	4.00	3.36	1.83	1.02	0.94	0.44	0.32	0.29	0.31
% error	**1.69**	**0.79**	**1.17**	**1.17**	**1.23**	**2.17**	**1.97**	**3.20**	**7.34**	**16.32**

Key: The highlighted values at the top of the table represent the emission wavelength, STDEV = standard deviation, % error = the standard deviation as a percentage of the mean.

Results in Table 4.2 show that the errors associated with the LS-5 spectrofluorimeter increase with increasing emission wavelength. This has been attributed to: (1) the fact that PMT sensitivity decreases with increasing wavelength, and (2) the smaller the signal, the greater the effect of background noise. This last point is of particular importance because it basically means that the errors associated with an individual EXEM spectrum are going to vary from sample to sample depending on the type and concentration of fluorophore present. However, as most coral solutions and the quinine sulphate solution used in Table 4.2 have similar fluorescent intensities

between 520-580 nm, a direct comparison can thus be made. At shorter wavelengths (i.e. between 400 nm and 520 nm), quinine sulphate fluorescence far exceeds coral solution fluorescence. In this case, the quinine sulphate errors are likely to be slightly lower than those associated with coral solutions although not by much since the difference between the quinine sulphate 520 nm percentage error and those between 400 nm and 520 nm was small.

Although these results can not be used as evidence to say anything about absolute fluorescent intensities, the errors associated with an individual EXEM spectrum were, with the exception of emissions above 580 nm, very low. Thus, although the absolute intensity of the fluorescent emissions may not be correct, the shape of the corrected EXEM spectrum most probably is.

Can EXEM spectra that were taken at different times be directly compared?: Two different time scales were considered, short-term variation (i.e. variation over an hour), and long-term variation (i.e. daily variation).

(a) **Short-term variation**: Using quinine sulphate, the fluorescent intensity of the 335/420 excitation/emission peak was measured six times every 20 minutes over a period of one hour.

Table 4.3 Variation in fluorescent intensity over an hour.

Repetition	0 (mins)	20 (mins)	40 (mins)	60 (mins)
1	655	675	670	671
2	655	670	670	670
3	655	665	670	670
4	650	660	665	679
5	650	660	665	679
6	645	660	665	678
Mean	652	665	668	675
STDEV	4.08	6.32	2.74	4.59
% error	**0.63**	**0.95**	**0.41**	**0.68**
Mean (X)	665			
STDEV (X)	9.63			
% Drift	**1.45**			

Key: STDEV = standard deviation, % error = the percentage error associated with an individual replicate set of six measurements, Mean (X) = the mean of all mean values; STDEV (X) = the standard deviation of the four mean values, % Drift = the percentage change in the mean fluorescent intensity value over the hour (calculated as the STDEV (X) as a percentage of Mean (X)).

Results in Table 4.3 show that although the error associated with an individual replicate set of six is relatively low (comparable to that in Table 4.2 which was 0.79%), mean fluorescent intensity actually increased over the hour. As fresh quinine sulphate was used for each measurement, this increase was attributed to instrumental drift which was calculated to be a fairly insignificant 1.45%. The fact that one EXEM spectrum can be produced in approximately 10 minutes means that the fluorescent intensity of bright and dull bands can be reliably compared if the analysis of one solution immediately follows the other.

(b) **Long-term variation**: This was not calculated using quinine sulphate as the fluorescence of this solution was found to decrease with time as a consequence of oxidation. Instead, long-term variations were calculated using the 355/400 nm DH_2O Raman scatter peak following the method of Coble *et al* (1993) (Raman scatter peaks are discussed in section 4.4.1). Replicate sets of six measurements were taken over two months.

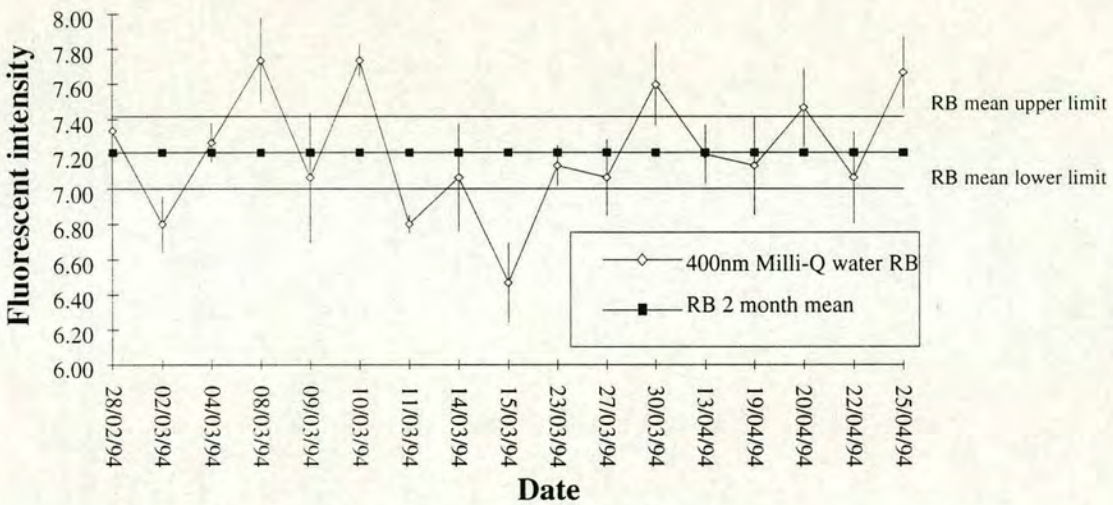

Figure 4.5 The fluorescent intensity of the 355/400 nm water Raman scatter peak taken over a two month period. The white diamonds represent the mean of six 355/400 nm measurements taken on a specified day. The errors associated with a replicate set of six are indicated as vertical error bars and range from 0.79% (11/3/94) to 5.23% (9/3/94). The black squares represent the two month Raman scatter peak mean value whose upper and lower limits were calculated using the mean % error which was 2.85% calculated from all the individual errors associated with a replicate set of six measurements (see Appendix A4.6).

The mean fluorescent intensity was calculated to be 7.2 (no units, see Appendix A4.6). Using the two month mean percentage error associated with a replicate set of six measurements (2.85%), Raman scatter peak mean upper and lower limits were calculated. Although no trend either to higher or lower fluorescent

intensities was observed over this period (see Figure 4.5), some of the mean values taken on specified days were outside the mean 355/400 nm upper and lower limits. Thus, although some of the variation seen was due to problems associated with measuring small fluorescent intensities, the LS-5 also appeared to impart a considerable amount of variation of its own (up to *ca.* 10%).

In conclusion, although it was considered reasonable to quantitatively compare the fluorescent intensities of EXEM spectra produced over relatively short periods such as an hour, a qualitative comparison should be adopted when comparing EXEM spectra produced over longer time scales than this.

4.3.4 Summary/ Key points:

(1) As fluorescent banding was first identified as a visible phenomenon, excitation wavelengths were selected to maximise visible fluorescence. For this reason, coral solutions were, in general, excited from 300 nm to 500 nm as the fluorophore groups thought to be responsible for fluorescence in the visible part of the spectrum were excited over this range. Because PMT sensitivity decreased with increasing wavelength, it was not possible to reliably record fluorescent emissions above 580 nm

(2) In general, the errors associated with an individual EXEM spectrum were small suggesting that the shape of an individual EXEM spectrum was reliable. This is of great importance when calculating the colour of fluorescent emissions as colour depends on the relative intensity of emission wavelengths rather than their absolute intensities (see section 4.5, pages 86-96, which deals with the colour of coral fluorescence).

(3) Instrumental shift over an hour is small enough to permit direct comparison of EXEM spectra taken within an hour or so. However, over longer periods of time, instrumental drift can be in excess of 10%. In these cases, relative rather than absolute fluorescent intensities should be compared.

4.4 THE FLUORESCENT CHARACTERISTICS OF CORAL SOLUTIONS:

4.4.1 Characteristic coral solution EXEM spectra: Two main excitation peaks were identified in 1-5M coral solution EXEM spectra; a dominant 330-345 nm excitation peak and the less intense 390 nm excitation peak (see Figure 4.6).

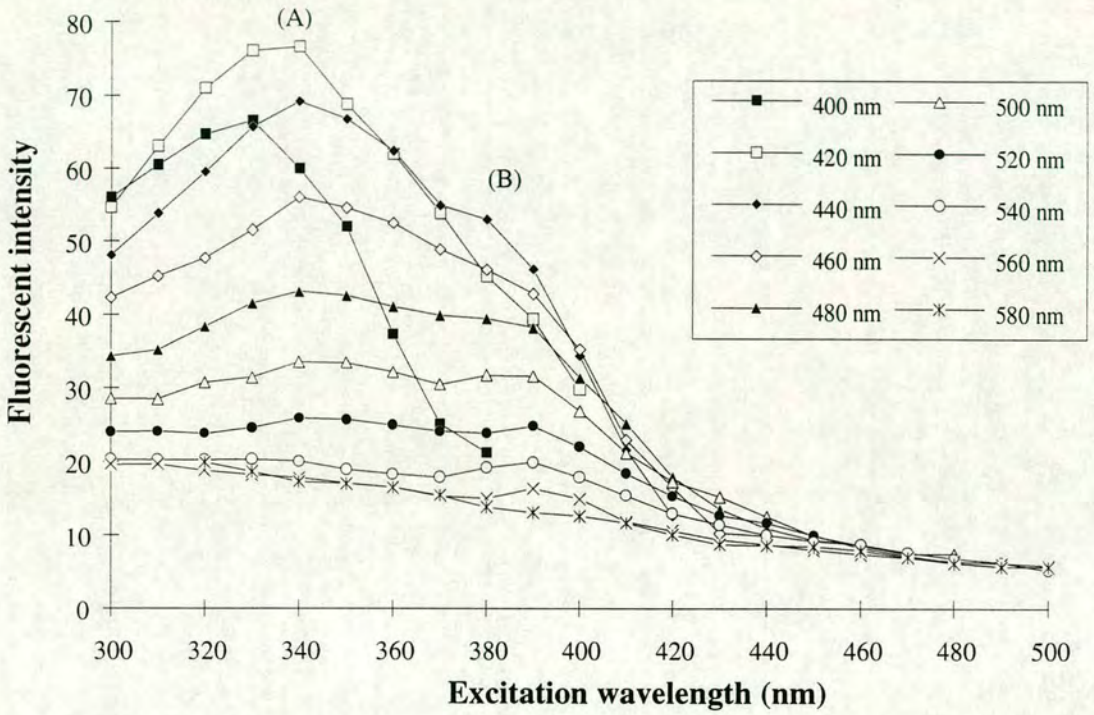

Figure 4.6 Characteristic coral solution EXEM spectra. 1M coral solution EXEM spectrum (from AQ-25-87 BB) showing the 330-345 nm and the 390 nm excitation peaks (A and B respectively).

In addition to these excitation peaks, a third very weak excitation peak at 275-285 nm was seen in some coral solutions (see Figure 4.7). Although Coble *et al* (1990) attributed this excitation peak to tryptophan, it could also be due to isoxanthopterin which has an excitation peak at 285 nm. Isoxanthopterin and tryptophan and are discussed in more detail in sections 4.8.1 and 4.8.2 respectively (see pages 132-140).

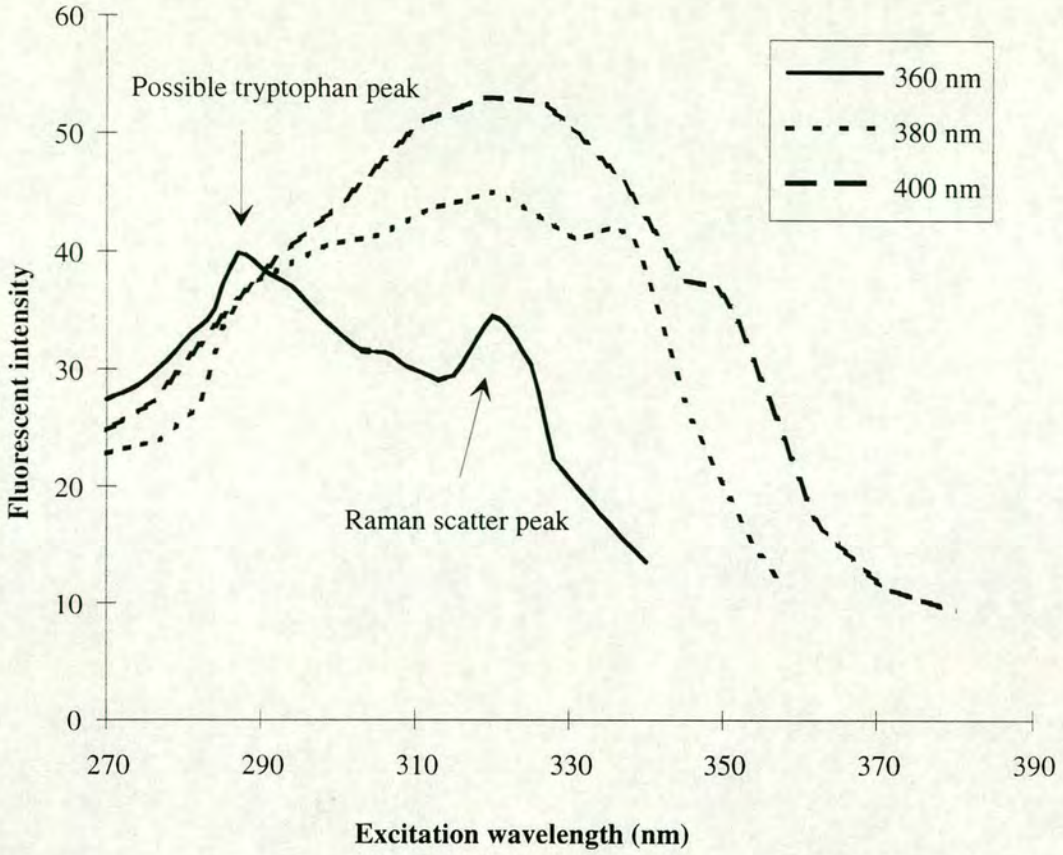

Figure 4.7 The 275-285 nm excitation peak seen in some dilute coral solutions. A full EXEM spectrum is not displayed because an excitation peak at 275-285 nm is not seen when the emission wavelength is greater than 380 nm.

Thus, it would appear that coral solutions contain two, possibly three, different excitation peaks. These peaks are attributed to different fluorophore groups i.e. fluorescing species with different (physical/chemical?) characteristics. Although each excitation peak could relate to a specific fluorophore (i.e. the 275-285 nm excitation peak could be due to either tryptophan or isoxanthopterin), they could also be due to a range of very similar fluorophores. This is much more likely to be the case for the 330-345 nm excitation peak as its is quite broad in comparison to the 390 nm excitation peak. The 330-345 nm excitation peak is the most dominant peak in dilute solutions (e.g. coral solutions) but this is not necessarily the case in very concentrated solutions. Possible reasons to account for this phenomenon are discussed in section 4.6.1, pages 97-114. Although this could be due in part to the LS-5 PMT being more sensitive to lower wavelengths, it could also be due to: (1) more 330-345 nm type fluorophores than 390 nm type fluorophores and/or, (2) the 330-345 nm type fluorophores are more fluorescent than the 390 nm type fluorophores.

As most 1M coral solutions were weakly fluorescent, small water Raman scatter peaks were sometimes seen (see Figure 4.7). When incident radiation is scattered, some of its energy is absorbed by the solvent molecules (water in this case). The scattered energy is therefore of a longer wavelength than the incident radiation which was why a Raman scatter peak appeared at 400 nm when DH_2O was excited at 355 nm (see Figure 4.8). As the amount of energy lost in this way is always constant, Raman scatter peaks appear separated from the incident radiation by the same frequency difference irrespective of the wavelength of the exciting light. Thus, every excitation wavelength will result in the formation of a Raman scatter peak, which is why, when EXEM spectra are produced for DH_2O, Raman scatter peaks can be seen to migrate (see Figure 4.8).

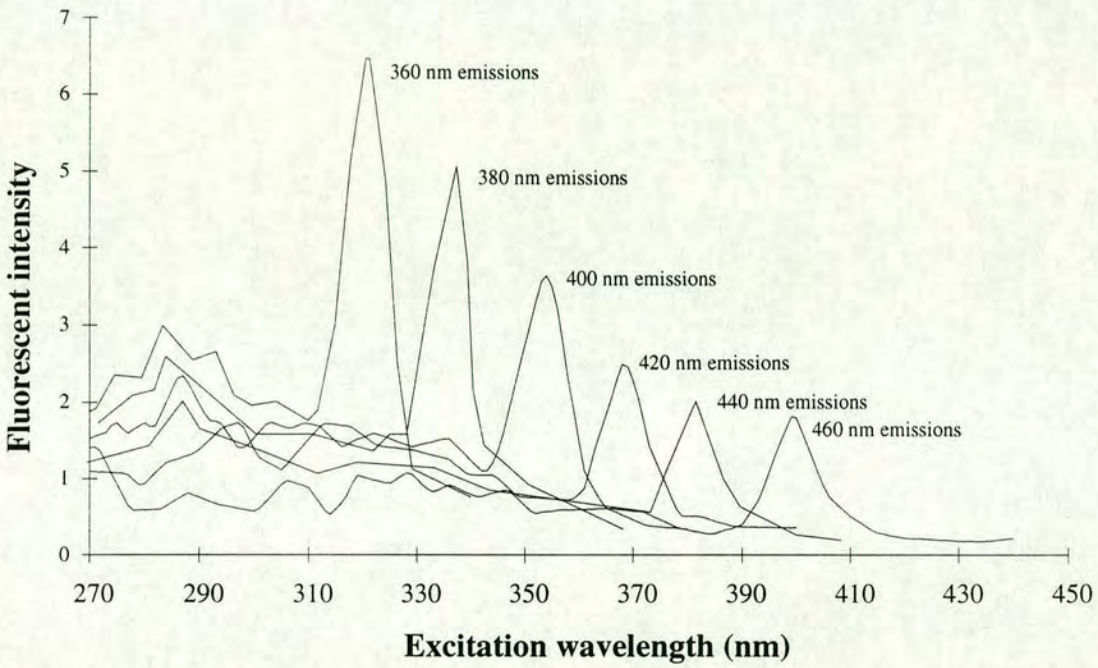

Figure 4.8 Water Raman scatter peaks.

The intensity of Raman scattering depends on the concentration and absorption characteristics of competing species in the solution. In general, the higher the concentration of competing species, the smaller the Raman scatter peaks. If the concentration of competing species is sufficiently high, Raman scatter peaks will not be seen. In other words, Raman scattering is a weak process that only occurs when the concentration of other molecules in solution is low. Although Mopper and Schultz (1993) applied a correction factor to account for Raman scattering (i.e. they subtracted Raman scatter peaks from their fluorescent signal), this was not done in this chapter because they were small enough to be considered insignificant.

4.4.2 Differences between bright and dull band EXEM spectra: EXEM spectra for 1M coral solutions from bright and dull bands are very similar (see Figure 4.9a and b).

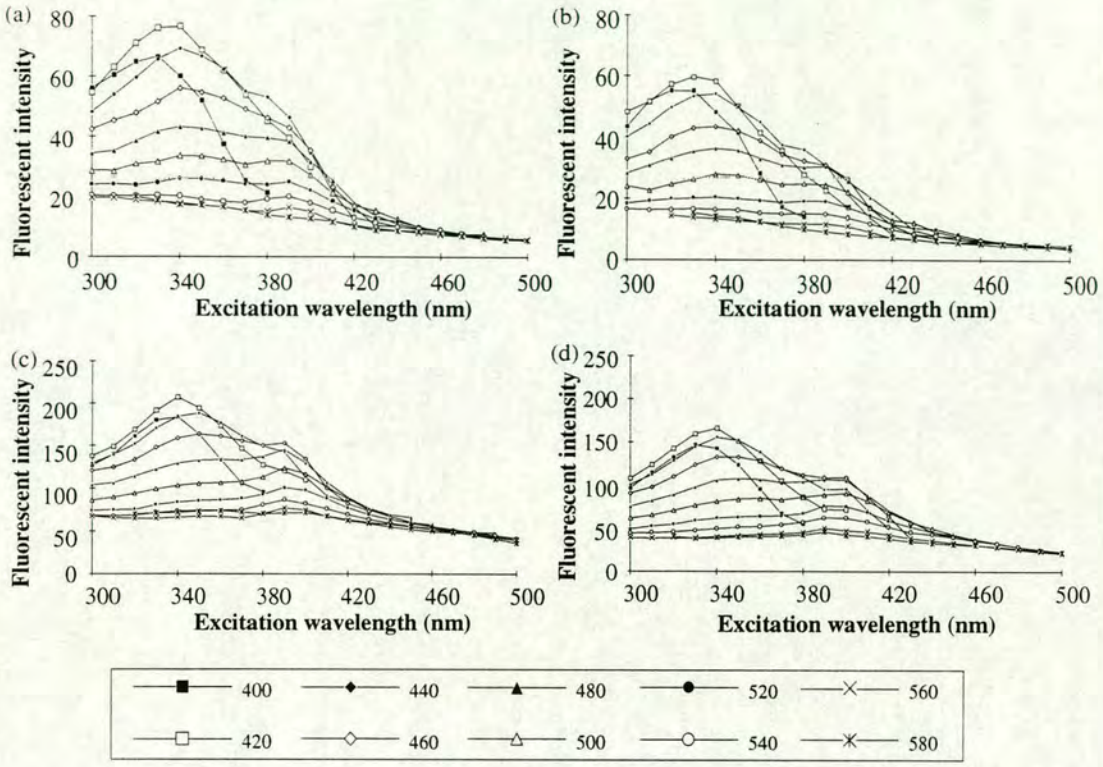

Figure 4.9 Bright and dull band EXEM spectra. (a) = a 1M bright band coral solution (AQ-25-87), (b) = a 1M dull band coral solution (AQ-25-87), (c) = a 2M bright band coral solution (PB-8-87), (d) = a 2M dull band coral solution (PB-8-87).

Even when more concentrated coral solutions were analysed (see Figure 4.9c and d), no significant difference in the shape of the EXEM spectra was detected between bright and dull bands from a particular sample. This suggested that the fluorophores responsible for bright and dull band fluorescence were the same or very similar.

In general, the main difference between 1M bright and dull band solutions was one of intensity, with bright bands being more intense than dull bands. This therefore suggests that bright bands have a higher concentration of fluorophores than dull bands which would be consistent with work by Susic *et al* (1991).

4.4.3 The fluorescent intensity of coral solutions from different locations: By examining the fluorescent intensity of coral solutions from different locations, it may be possible to determine whether the supply of fluorophores in the marine environment is spatially heterogeneous.

Methods and materials: 1993 reef front *Porites lutea* from the Aquarium Reef (on the SW shore) and Porites Bay Reef and Tin Smelter Reef (on the NE shore) were used to compare the fluorescent intensity of corals exposed to the SW and NE monsoons respectively. Although no attempt was made to sample individual fluorescent bands, samples were prepared from bands which were deposited during the same five year time period. 2M coral solutions, taken from the axis of maximum linear extension, were prepared in the manner described in section 4.2.5.

Results: In general, the fluorescent intensities of corals growing on the NE side of the peninsula were greater than those growing on the SW side (see Figure 4.10).

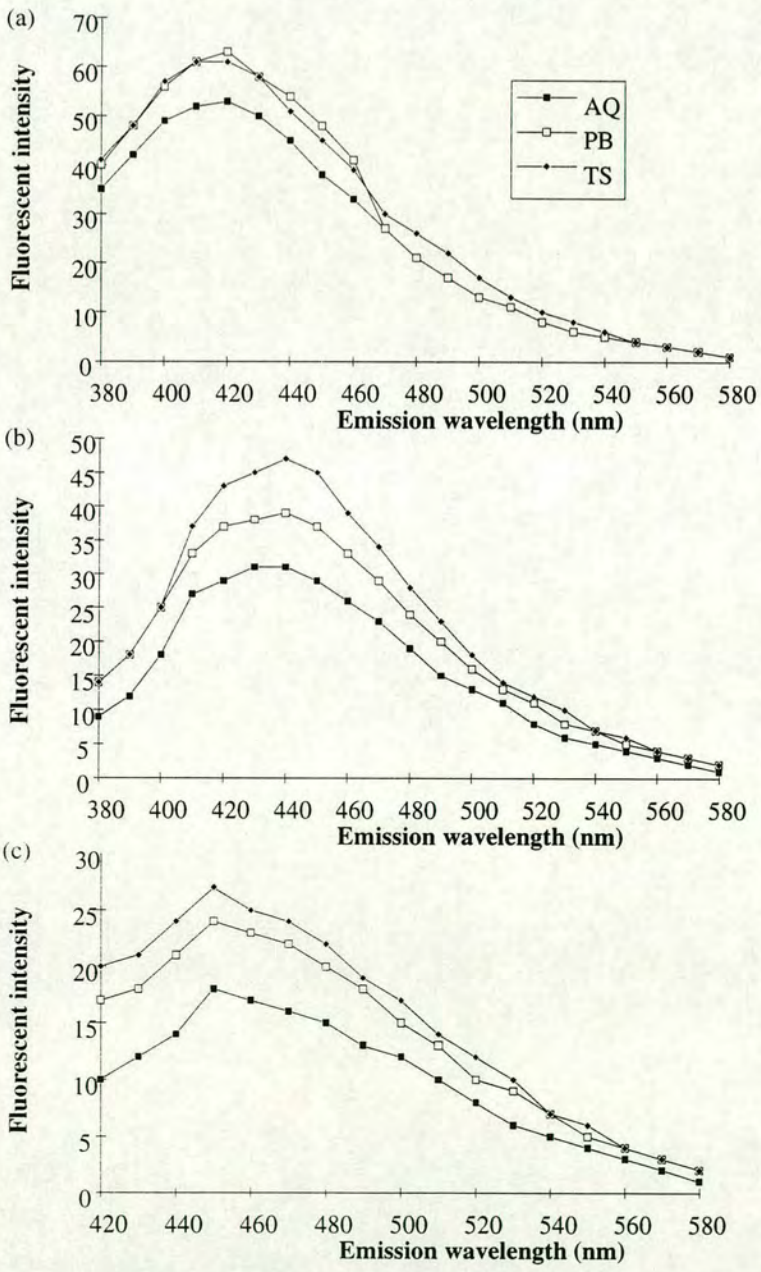

Figure 4.10 Emission spectra of corals collected around the peninsula. TS = Tin Smelter Reef, PB = Porites Bay Reef (both on the NE side of the peninsula) and AQ = Aquarium Reef (on the SW side of the peninsula; (a) 330 nm emission spectrum; (b) 360 nm emission spectrum; (c) 390 nm emission spectrum.

These results therefore suggest that the concentration of fluorophores/g of coral is greater on the NE side of the peninsular than on the SW side. Although the reason for this relationship was not absolutely determined, it suggests that the concentration of fluorophores in the seawater around Ko Phuket is spatially heterogeneous.

4.4.3 Do humic acids contribute to the fluorescence of *Porites lutea* from Ko Phuket?: As Boto and Isdale (1985) and Susic *et al* (1991) identified humic acids as the main fluorescent constituent of coral skeletons, two industrially prepared humic acid solutions were tested to see whether their fluorescent properties were similar to those found in *Porites lutea* from Ko Phuket. One solution was made from Aldrich humic acid and the other from Fluka humic acid. A known amount of each was dissolved in a litre of DH_2O. This was then filtered through pre-weighed 0.45 μm Whatman filter paper to remove any undissolved humic and inorganic material. These filter papers were dried and re-weighed and the amount of insoluble material was determined. This was subtracted from the initial amount of humic acid added to enable the concentration of each solution to be calculated. The following concentration Aldrich humic acid solutions were used in this chapter: 172 ppm, 86 ppm, 43 ppm, 17 ppm and 9 ppm. The following concentration Fluka humic acid solutions were used in this chapter: 144 ppm, 72 ppm, 36 ppm, 14 ppm and 7 ppm. At concentrations below 20 ppm, the industrial humic acid EXEM spectra were similar to those of 1M coral solutions (see Figure 4.6 on page 79, and Figure 4.9 on page 82, for typical coral solution EXEM spectra; and Figure 4.11a for a low concentration humic acid EXEM spectrum).

The major difference between industrially prepared humic acid solutions and coral solutions is that the former contains a 470 nm excitation peak which becomes more dominant as concentration increases (see Figure 4.11b). The reasons for the differences between (a) and (b) in Figure 4.11 are discussed in section 4.6.1, pages 97-114. Thus, it would appear that the fluorescent characteristics of low concentration industrial humic acid solutions and 1M coral solutions are very similar which is compatible with the hypothesis that humic acids are the dominant fluorophores in *Porites lutea* from Ko Phuket.

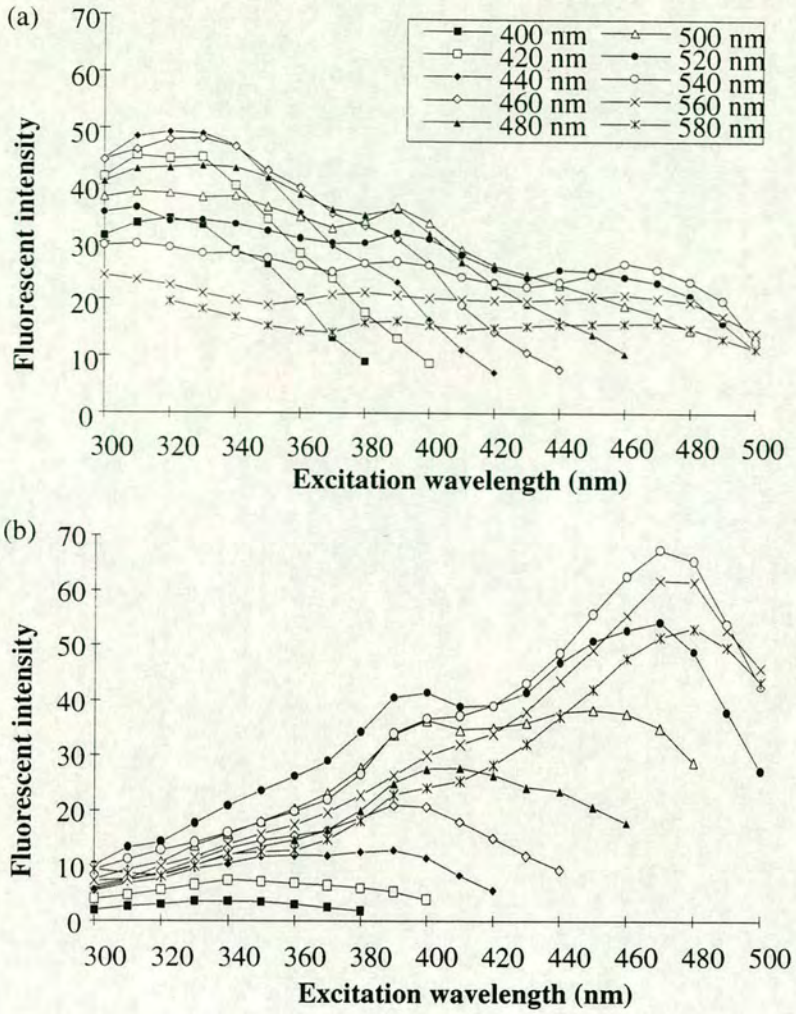

Figure 4.11 EXEM spectra of (a) 14 ppm Fluka humic acid solution and (b) 144 ppm Fluka humic acid solution.

4.4.5 Summary/Key points: The results of this section can be summarised as follows:

(1) Coral solution EXEM spectra are characterised by two main excitation peaks, a dominant 330-345 nm excitation peak and a less intense 390 nm excitation peak.

(2) No significant differences in the shape of bright and dull band EXEM spectra were detected which suggests that the source or sources responsible for both band types are the same or very similar.

(3) The main difference between bright and dull band coral solutions EXEM spectra appears to be one of intensity, with bright bands being more intense than dull bands. This therefore suggests that the concentration of fluorophores in bright bands is higher than in dull bands.

(4) Corals growing on the NE side of the peninsula were more fluorescent than those growing on the SW side of the peninsula. This could reflect a greater abundance of fluorophores in the marine environment on the NE side of the peninsula.

(5) The fact that coral solution EXEM spectra were so similar to the EXEM spectra produced for low concentration industrially prepared humic acid solutions suggests that humic acids are probably the dominant fluorophores in the Thai study area.

4.5 THE COLOUR OF FLUORESCENCE:

Because the colour of fluorescence is discussed extensively throughout this and subsequent chapters, it is appropriate to introduce the topic here.

4.5.1 Predicting the colour of coral solution fluorescence using EXEM spectra: EXEM spectra do not account for the sensitivity of our eyes to different wavelengths of light and, therefore, can not be used directly to predict what colour will be seen. The eye's response to different wavelengths of visible light is not a uniform one. Figure 4.12 shows that the eye's maximum response is at 550 nm which is in the green/yellow part of the spectrum.

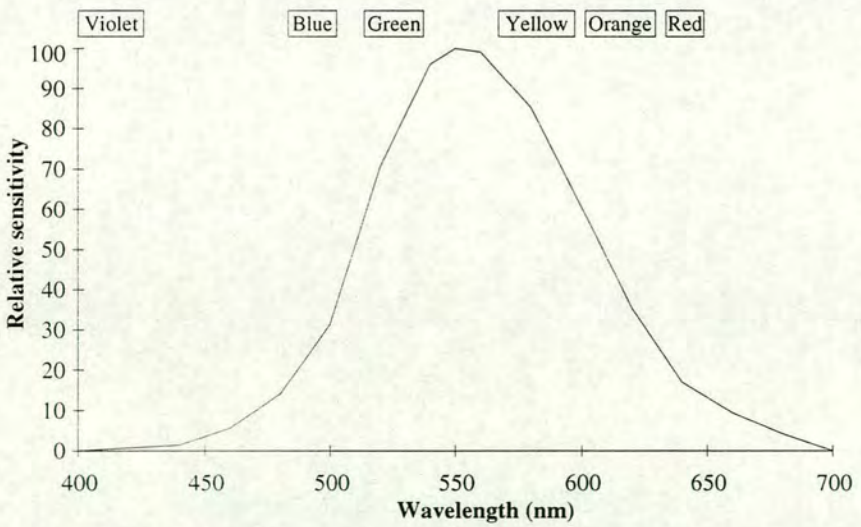

Figure 4.12 The general eye response curve (Bergmans 1960).

Our ability to see blue and red objects is rather limited and depends on whether the source is monochromatic (single source) or polychromatic (many sources). If it is polychromatic, the ratio of blue or red light to green/yellow light has to be exceedingly large if either blue or red light is to be seen. The light sensitive organs in our eyes possess three types of colour sensitive receptor known as the red, blue and green receptors. Each type of receptor has its own way of evaluating the light it receives. For example, the red receptors have a certain conversion factor (X factor) for each spectral light type (see Figure 4.13).

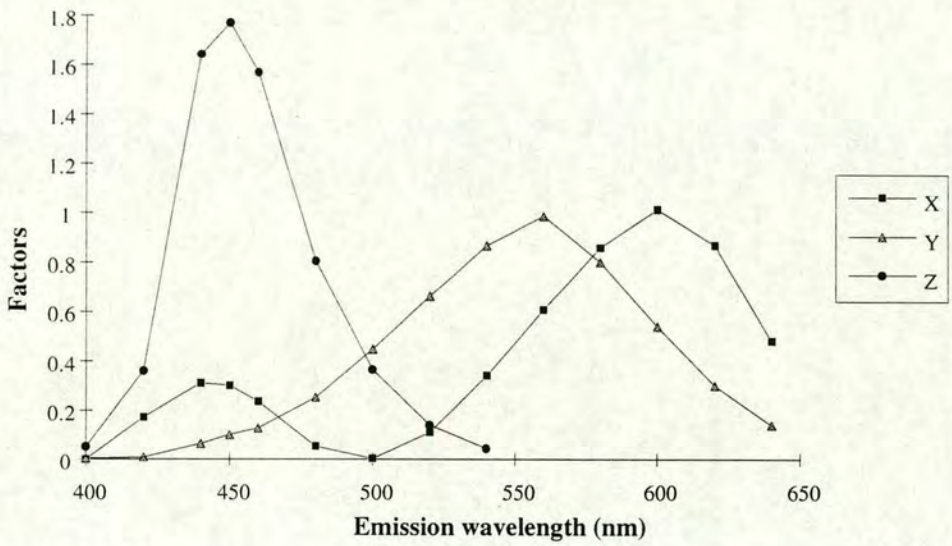

Figure 4.13 X, Y and Z conversion factors (Bergmans 1960).

When this conversion factor is applied, the red value for each spectral light type can be determined. Adding all the individual red values together gives the total red value of the composite light known as the red component (X). The green (Y) and blue (Z) components are calculated in exactly the same way. Although the three types of receptor in the eye predominantly absorb the wavelengths they are named after, they do not absorb those wavelengths exclusively. For example, the red component includes purple wavelengths (see Figure 4.13). An important feature in the prediction of colour is that when the amounts of red, blue and green light are all increased in equal proportions, the colour according to the eye remains unchanged even though the total brightness increases. This is because colour according to the eye is due to the relative amounts of blue, green and red light reaching the receptors rather than the absolute amounts of each component. In other words, colour is independent of intensity.

ype (x, y and z) are determined as follows:

$$\frac{Z}{+Y+Z)}$$

ange between 0 and 1, the sum of x, y and z is
calculated, the colour seen by the eye can be
iangle which was officially accepted by the
ation (I.C.I.) in 1931 as a means of colour

Officially accepted by the International Committee on
olour determination.

For further details concerning the manner with which colour is perceived see Bergmans (1960).

4.5.2 The excitation wavelengths used to predict colour: Traditionally, coral samples have been excited with longwave (~360 nm) u/v light (Isdale 1984; Boto and Isdale 1985; Smith *et al* 1989; Klein *et al* 1990). Although, up to three main excitation peaks were detected in industrial humic acid (330-345 nm, 390 nm and 470 nm) and two in coral solutions (330-345 nm and 390 nm), none of these corresponded to an excitation wavelength of 360 nm. Thus, exciting at 360 nm will not maximise fluorescent emissions, and will not make all the fluorophore groups fluoresce. For example, exciting at 390 nm will mean that all molecules whose excitation peaks occur at shorter wavelengths will not be excited (longer wavelength fluorophore groups may be excited as a consequence of energy transfer, see section 4.6). As the eye is best at detecting light in the green/yellow part of the spectrum, it is important to find an excitation wavelength that maximises fluorescence in this region. Choosing the right excitation wavelength to maximise green/yellow emission also depends on the concentration of fluorophores in the solution (discussed in section 4.6.1). As a consequence, it is very unlikely that any one excitation wavelength will maximise fluorescent emissions in the green/yellow part of the spectrum for all coral solutions. For these reasons, the colour of coral solution fluorescence was examined at three excitation wavelengths, 330 nm, 360 nm and 390 nm while the colour of Fluka and Aldrich humic acid fluorescence was also examined at 470 nm as this excitation peak was well developed in the more concentrated solutions. Calculations have shown that the predicted colour of coral fluorescence depends, amongst other things, on the wavelength of light used to excite the sample (see Figure 4.15). As excitation at a certain wavelength will produce fluorescence at a longer wavelength, the longer the excitation wavelength, the longer the emission wavelengths. For example, when coral solutions are excited at 440 nm, the fluorescence is emitted at wavelengths greater than approximately 460 nm (i.e. wavelengths between 400-460 nm will not be emitted). Thus, as far as colour is concerned, there is a higher proportion of longer wavelength emissions (i.e. green and red light). For this reason, when the colours of fluorescence were compared, they were always done so at the same excitation wavelength.

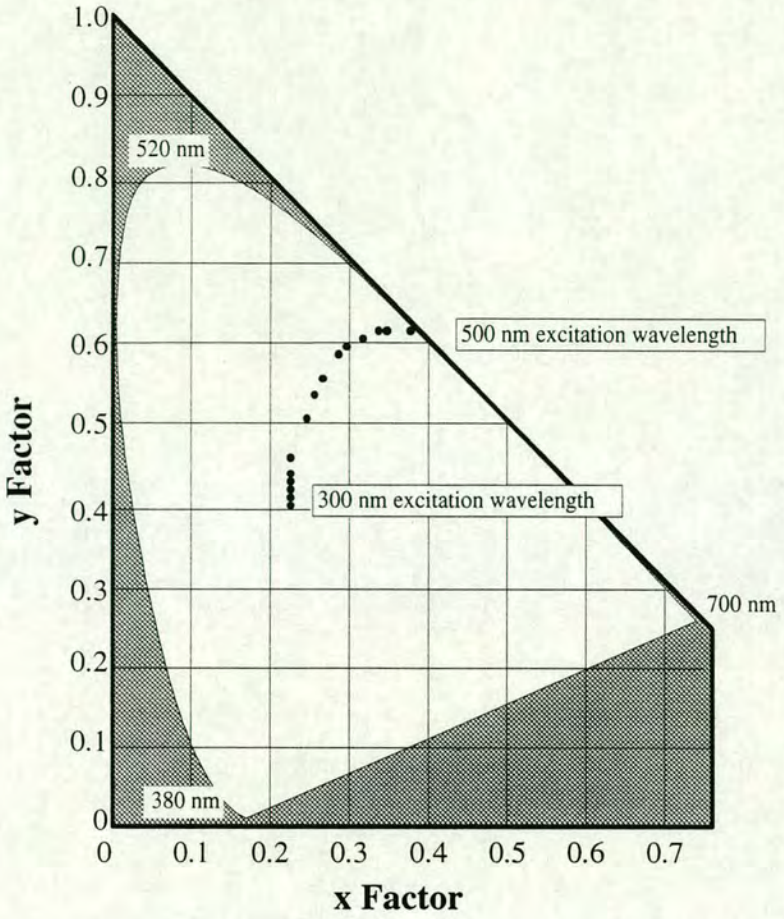

Figure 4.15 The change in the predicted colour of fluorescent emissions with increasing excitation wavelength for a 144 ppm Fluka humic acid solution plotted on an I.C.I. colour chart. A shift towards higher wavelength emissions is seen in undiluted Fluka humic acid as the excitation wavelength is increased. Refer to Figure 4.14 on page 88 to assign colours to these points.

The effect LS-5 errors have on the predicted colour of coral fluorescence will increase with increasing excitation wavelength. This is because, as the excitation wavelength increases, proportionally more green/yellow fluorescence will be emitted. As our eyes are very receptive to light in this region, EXEM variation brought about by the LS-5 spectrofluorimeter (which is considerable in this part of the spectrum) could have a large effect on the predicted colour of fluorescence. The errors associated with the predicted colour of fluorescence were estimated for each emission wavelength using the quinine sulphate short-term errors given in Table 4.2 on page 75. When upper and lower limits were calculated for a 144 ppm Fluka humic acid solution, results showed that changes in the colour of fluorescence (exciting at 330 nm, 360 nm, 390 nm and 470 nm) were insignificant (see Figure 4.16). In other words, although the errors associated with the measurement of fluorescent emissions were at a maximum (560-580 nm) in part of the visible

spectrum that the eye sees effectively, they did not result in a significant difference in the predicted colour of fluorescence.

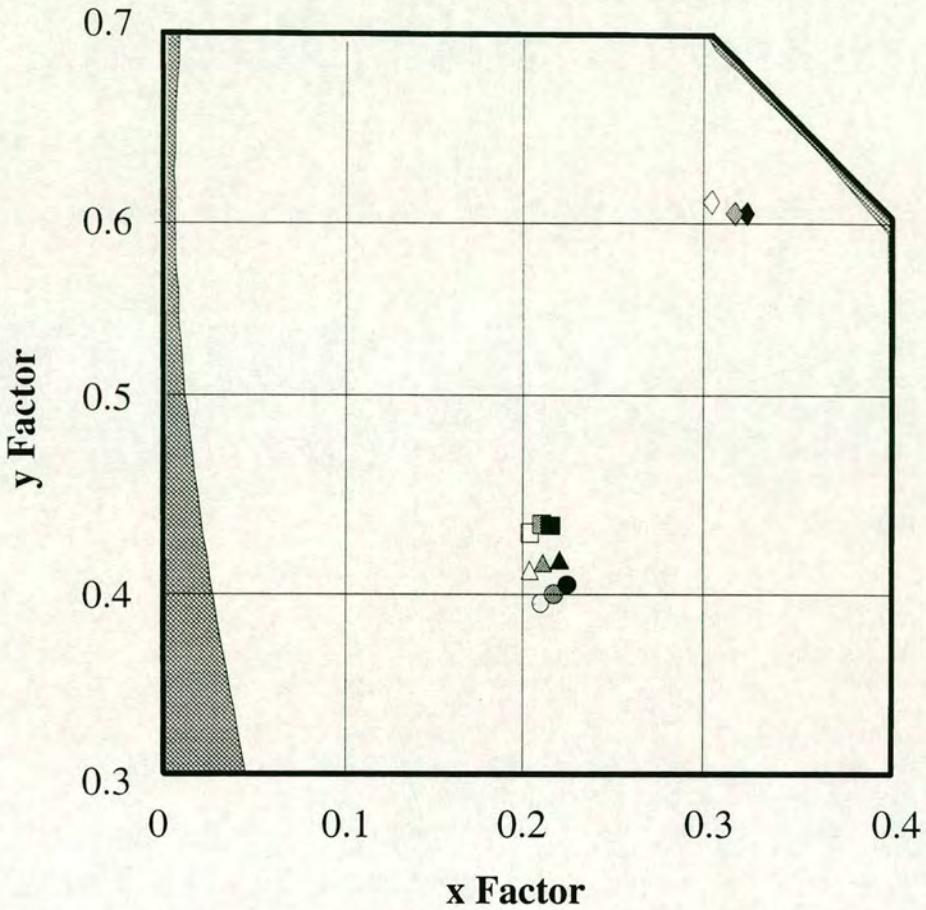

Figure 4.16 The effect that LS-5 errors have on the predicted colour of fluorescence. 144 ppm Fluka humic acid used in this calculation. Key: Circles = 330 nm excitation wavelength, triangles = 360 nm excitation wavelength, squares = 390 nm excitation wavelength, diamonds = 470 nm excitation wavelength, white colour = lower limit, grey = predicted colour of fluorescence, black = upper limit. Refer to Figure 4.14 on page 88 to assign colours to these points.

4.5.3 Why the LS-5 predicted colour of fluorescence does not correspond to the colour of fluorescence seen under the u/v lamp: When a 144 ppm Fluka humic acid solution was viewed under a u/v lamp with a nominal emission wavelength of 370 nm (see Chapter 2, Figure 2.15, page 42), a green/yellow colour was seen. However, its predicted colour of fluorescence when excited at 370 nm using the LS-5 spectrofluorimeter was dark green. It is possible that this discrepancy is due to the fact that the u/v lamp emits radiation over a large range of wavelengths. Although emissions were centred on 370 nm, a significant amount of radiation was emitted between 330 nm and 430 nm. Thus, using LS-5 EXEM data, the X, Y and Z factors

were calculated at each excitation wavelength over this range assuming that the incident radiation was not a limiting factor (i.e. the fluorophores were saturated with u/v light). This was a reasonable assumption to make considering the solutions examined in this chapter were not extremely concentrated. This assumption does not apply to extremely high concentration solutions as all the exciting light is absorbed over a very short distance so that the molecules in the body of the solution are not excited. This is clearly not the case even when 144 ppm Fluka humic acid solutions are used as the exciting light can be seen to pass through the solution. The values obtained in this way were then added together to enable the x, y and z values to be determined and hence the colour that would be produced by exciting a 144 ppm Fluka humic acid solution with a number of different wavelengths. Although a slight shift to longer wavelengths was predicted, it was not enough to account for the visual difference between predicted and seen (see Appendix A4.7). When the 144 ppm Fluka humic acid solution was moved closer to the u/v lamp it appeared to change colour, becoming yellower as the distance was reduced. This suggested that the fluorophores were under-saturated with respect to at least some (but possibly all) of the excitation wavelengths. The closer the humic acid solution was to the u/v lamp, the greater the intensity of those unsaturated wavelengths and the greater their effect on the colour of fluorescence seen. The X, Y and Z factors were recalculated at each excitation wavelength over this range taking into account the intensity of the u/v lamp at each excitation wavelength. The values obtained in this way were then added together to enable the x, y and z values to be determined and hence the colour that would be produced by exciting the 144 ppm Fluka humic acid solution with a number of different wavelengths of varying intensity. However, when the colour predicted in this way was compared to the colour predicted by exciting at 370 nm only, no significant difference was detected (see Appendix A4.8). Therefore, while the fact the u/v lamp emits over a range of wavelengths may account for some of the difference in colour between predicted and seen, it would not appear to be the only factor.

Another, and perhaps more important, reason why the colour of fluorescent emissions predicted using the LS-5 spectrofluorimeter did not correspond to what was seen under the u/v lamp could be because the LS-5 only recorded emissions up to 580 nm for the reasons given in section 4.3.2 (pages 72-74). This meant that a large part of the visible spectrum was ignored (580-700 nm). Even though work by Boto and Isdale (1985; also see Chapter 1, Figure 1.3, page 6) suggested that bright and dull band fluorescent emissions between 580-700 nm were weak compared to

emissions in the rest of the visible spectrum, the fact that emissions were not recorded in this region means that the colour of coral fluorescence predicted using LS-5 data is likely to be bluer than in reality.

Light scattered/reflected off the surface of the coral could be another reason why the colours predicted using the LS-5 spectrofluorimeter and those seen under the u/v lamp did not coincide. As mentioned earlier, the u/v lamp emits radiation in the purple part of the visible spectrum. Any light scattered/reflected off the coral surface will therefore be seen along with the fluorescent emissions. Although this would tend to shift the colour of fluorescence observed by the eye towards shorter emission wavelengths, scattered/reflected light could, nevertheless, result in differences between predicted and seen.

4.5.4 The predicted colour of bright and dull band fluorescence: When bright and dull band emission spectra (330 nm, 360 nm and 390 nm) were compared, the biggest differences generally occurred between 420 nm and 440 nm (see Figure 4.17 a, c and e). In other words, bright bands emitted proportionally more blue light than dull bands. However, this is not what the eye sees and when the data were adjusted to account for the eye's response to colour (using the general eye response curve in Figure 4.12 on page 86), the maximum difference between bright and dull bands occurred between 520 nm and 540 nm (see Figure 4.17b, d and f). As the errors associated with the measurement of fluorescent emissions between 400 nm and 520 nm were quite small (see Table 4.2, page 75), the 520-540 nm peak produced when bright band eye adjusted data was subtracted from dull band eye adjusted data was likely to be real. As far as fluorescent emissions above 560 nm are concerned, the large errors associated with their measurement (as indicated by the shaded area) suggest that the eye adjusted BB-DB data in this region is rather unreliable and not to be trusted.

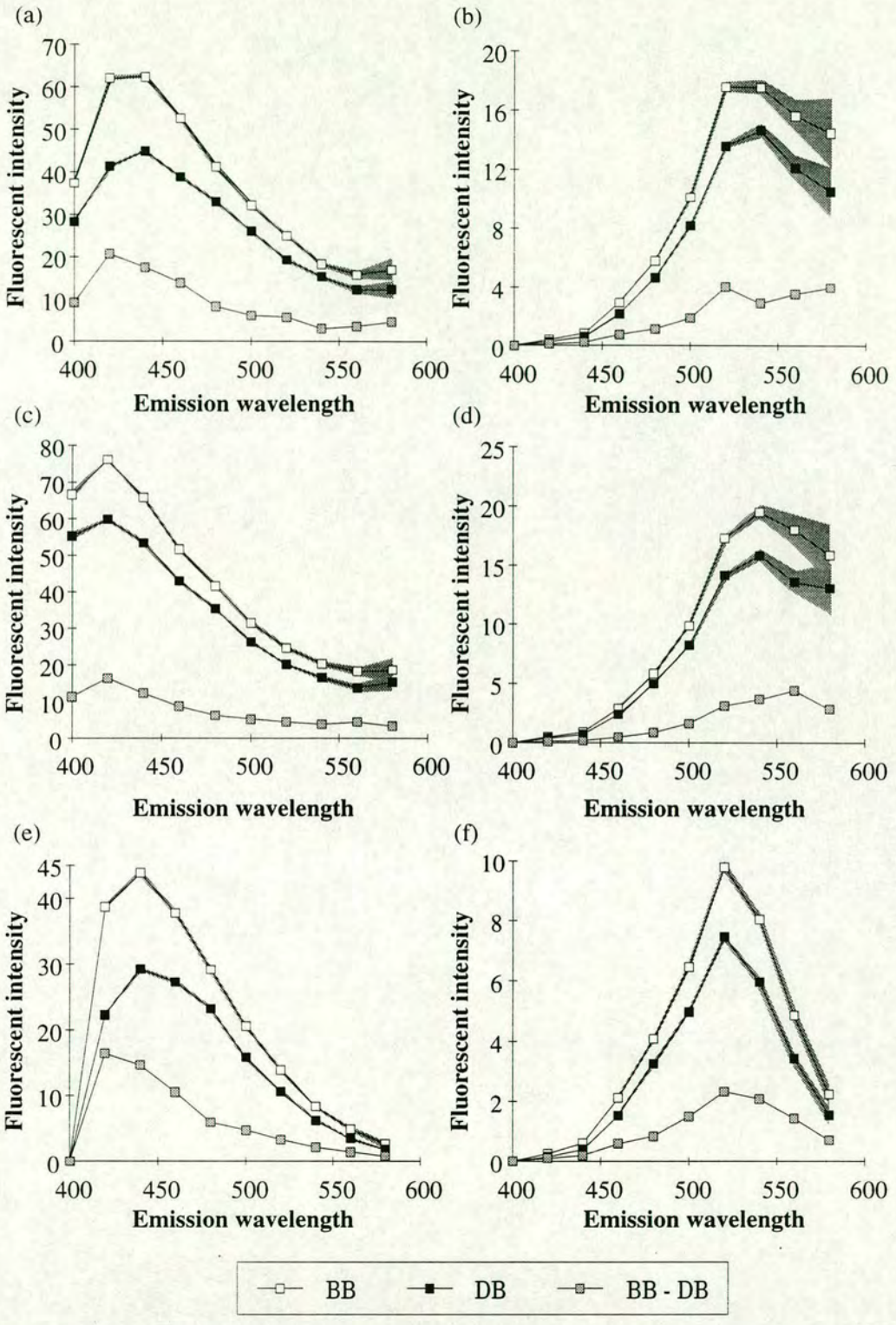

Figure 4.17 Bright and dull band 330 nm (a and b), 360 nm (c and d) and 390 nm (e and f) emission spectrum for AQ-25-87. (a, c and e) not adjusted to eye response (b, d and f) adjusted to eye response. BB = bright band; DB = dull band; BB - DB = the difference between bright and dull band fluorescent intensity. The shaded areas represent the errors associated with measurements at their respective emission wavelengths (values taken from Table 4.2 on page 75). Please pay close attention to the Y axis scale as they vary.

Although results suggested that bright bands emitted proportionally more fluorescence in the green part of the visible spectrum (520 nm) once emissions had been adjusted to account for the eye, the predicted colour of 1M coral solutions was blue when they were excited at 330 nm, 360 nm and 390 nm. Figure 4.18 shows the predicted colour of fluorescence when 1M bright and dull band solutions were excited at 360 nm.

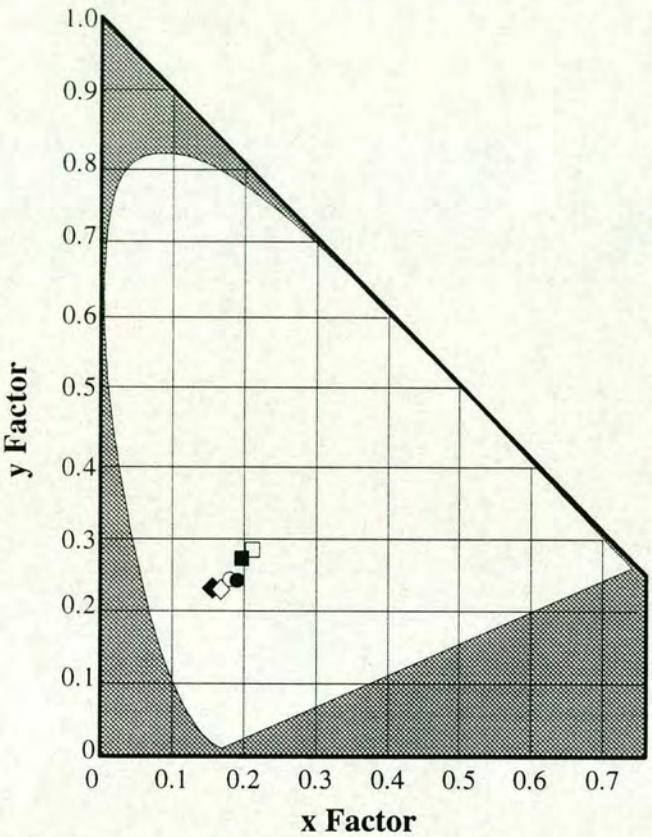

Figure 4.18 The predicted colour of fluorescence of 1M coral solutions. Key: white = bright bands, black = dull bands, diamonds = AQ-25-87, circles = TS-2-93, squares = TS-9-87. Refer to Figure 4.14 on page 88 to assign colours to these points.

The fact that bright and dull bands both emit the same colour of fluorescence adds further weight to the hypothesis that the fluorophores responsible for bright and dull band fluorescence are the same or very similar, i.e. both types of fluorophores are found in each band. This is in contrast to work by Boto and Isdale (1985) who suggested that bright band fluorescence is brought about by the presence of terrestrial humic acids which are not found in dull bands. Although results in this section suggest that the same types of fluorophores cause bright and dull band fluorescence, it is not possible at this stage to comment on the origin of the fluorophores.

4.5.5 Summary/Key points:

(1) The predicted colour of coral fluorescence depends to some extent on the wavelength of light used to excite the coral solution. As the excitation wavelength increases, the predicted colour of fluorescence shifts to longer wavelengths. For this reason, when comparing the predicted colour of fluorescence of one sample with another, it should always be done at the same excitation wavelength. The predicted colour of coral fluorescence was calculated at three excitation wavelengths, 330 nm, 360 nm and 390 nm. 330 nm and 390 nm were chosen as they corresponded to the two main excitation peaks in coral solutions thereby maximising fluorescent emissions. 360 nm was chosen as this excitation wavelength is commonly used in coral fluorescent studies.

(2) Discrepancies between the predicted colour of coral fluorescence and that seen under a u/v lamp have been attributed to the following: (1) the inability of the LS-5 spectrofluorimeter to record emissions over the whole of the visible spectrum, (2) the fact that the u/v lamp emits a considerable amount of light over a wide range of wavelengths, and (3) scattered/reflected light from the coral surface.

(3) Variations in the shape of coral emission spectra below 450 nm have very little effect on the final colour predicted, as the eye is unable to record light in this region effectively.

(4) Although the maximum difference between bright and dull band eye-adjusted data appeared to occur in the green part of the spectrum (520 nm), it was not large enough to produce a difference in the predicted colour of fluorescence. This could imply that bright and dull bands contain the same or very similar types of fluorophores.

4.6 VARIOUS FACTORS THAT COULD AFFECT THE FLUORESCENCE OF CORAL SOLUTIONS:

The abundance of fluorophores in coral solutions made from the same mass of coral skeleton varied considerably between samples. Thus, many of the experiments carried out in this section would be specific to a particular sample and not generally applicable to all coral solutions. Large amounts of material were required to study the effects of concentration, for repeat experiments and because coral fluorescence diminished with time. Thus, a source was required which fulfilled the following criteria: (1) its fluorescent characteristics were similar to coral fluorescence; (2) its concentration could be calculated thus enabling a large number of experiments to be conducted under identical conditions of concentration, and (3) a large common stock was available. Therefore, Aldrich and Fluka industrial humic acid were used.

4.6.1 The effect fluorophore concentration has on the fluorescence of humic acid and coral solutions: "The fluorescent properties of multi-fluorophore systems strongly depend on concentration as a result of the following:

(a) **Inner filter effects:** These relate to the absorption of light by non-fluorescent impurities and can affect the coral fluorophores in two ways. Firstly by absorption of the exciting light such that it is prevented from reaching the target fluorophores. Thus, as the concentration of these species increases, fluorescence at all emission wavelengths will decrease. Secondly, via the re-absorption of fluorescence. This process is known as radiative energy transfer (RET) and in the particular case where the absorbing and emitting species are identical it is termed self-absorption.

(b) **Quenching:** This is a general term used to describe intermolecular processes which compete with fluorescence and includes complex formation and non-radiative energy transfer (NRET). Static and dynamic quenching are the two types of quenching process that involve complex formation. In static quenching, the fluorescent molecule in its ground state interacts with other molecules to form an aggregate which constitutes a new light absorbing species. In dynamic quenching, the fluorescent molecule, in its excited state, interacts with other molecules to form an excited complex with its own fluorescent properties. These two quenching states may involve interaction with molecules of the same species (concentration quenching) or with those of different species

(impurity quenching). NRET involves the transfer of excitation energy from the donor to the acceptor without the emission of a photon. For this to happen, the absorption spectrum of the acceptor has to overlap with the emission spectrum of the donor. The greater the amount of overlap the greater amount of quenching that can occur. The energy is transferred as a result of either long range (up to 10 nm) or short range (touching) interactions between donor and acceptor molecules." Matthews *et al* in review.

The aim of this study was to determine how the fluorescent properties of industrial humic acid and coral solutions (both multi-fluorophore systems) are affected when the concentration of all fluorophores in the solution are increased without changing the relative concentration of each fluorophore in that solution. In other words, changing the absolute concentration of fluorophores without changing their relative concentrations.

Methods and materials: In order to determine the effects of concentration, EXEM spectra were produced for the following concentration Fluka humic acid solutions: 144 ppm, 72 ppm, 36 ppm, 14 ppm and 7 ppm. All pH's were adjusted to 9.

Results and discussion: Although this is not the first study to discuss the effect that concentration has on the fluorescent properties of industrial humic acid and coral solutions (see Susic and Boto 1989), this is the first study which details the nature of these changes.

The effect concentration has on the colour of coral fluorescence: Because both coral solutions and industrial humic acid are multi-fluorophore systems in which the type and concentration of fluorophores (either collectively or as individual groups) and their relations to each other are unknown, it is impossible to tell exactly what happens when the concentration is altered. Bearing this in mind, results show that as the concentration of Fluka humic acid increases, the 470 nm excitation peak becomes more dominant (see Figure 4.11 on page 85) and fluorescent emissions shift towards longer wavelengths (i.e. from blue to green/yellow) (see Figures 4.19 and 4.20).

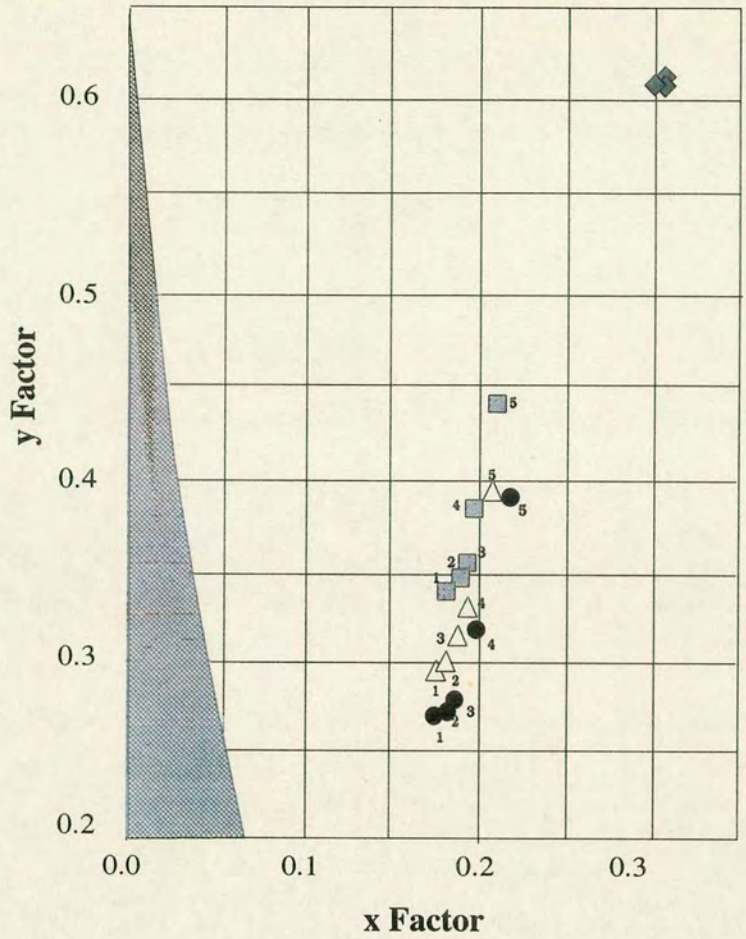

Figure 4.19 The effect concentration has on the colour of Fluka humic acid at 330 nm, 360 nm, 390 nm and 470 nm. Key: circles = excitation at 330 nm; triangles = excitation at 360 nm; squares = excitation at 390 nm; diamonds = excitation at 470 nm. Concentration increases from 1 (7 ppm) to 5 (144 ppm) at each excitation wavelength. Refer to Figure 4.14 on page 88 to assign colours to these points.

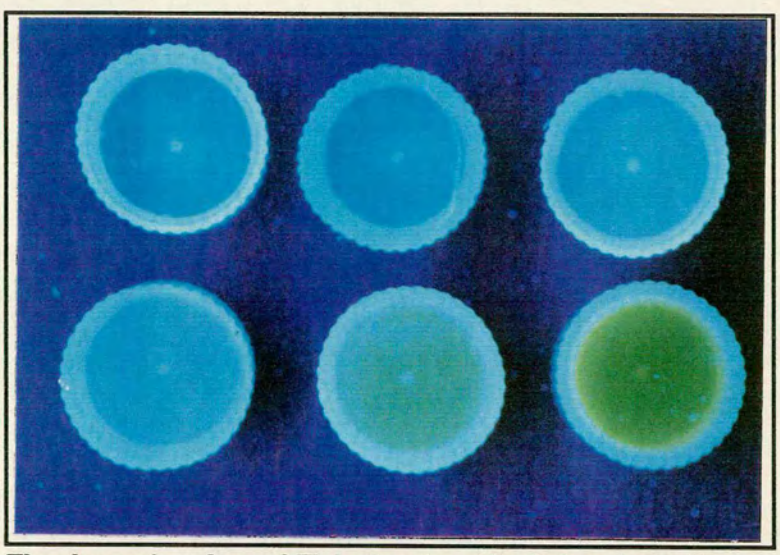

Figure 4.20 The change in colour of Fluka humic acid solutions brought about by increasing concentration. From top left to bottom right: DH_2O, 7 ppm, 14 ppm, 36 ppm, 72 ppm and 144 ppm.

These results have been attributed in part to the effects of energy transfer (ET). During ET, energy is transferred from molecules that absorb high energy wavelengths to those that absorb lower energy wavelengths. For example, fluorophores that were excited at 330 nm may transfer some of their energy to molecules that absorb at or around 390 nm. These molecules may then emit some of this energy as fluorescence at longer wavelengths. However, this does not mean that the fluorescent intensity of the 390 nm excitation peak group will increase at the expense of the 330 nm excitation peak group. This is because any fluorescence that is emitted as a consequence of exciting the sample at 330 nm, whether it is due to fluorophores that were excited directly at this wavelength or to fluorophores that were excited as a consequence of energy transfer (i.e. the 390 nm fluorophores), will be recorded as the fluorescent emissions produced when the sample was excited at 330 nm. In other words, if energy transfer does occur, the intensity of fluorescence at 330 nm could relate to more than one fluorophore group. Although, the 390 nm excitation peak fluorophores may contribute to the fluorescence of the 330 nm peak fluorophores when the sample is excited at 330 nm, this will not affect the size of the 390 nm excitation peak when the sample is excited at 390 nm. The size of the 390 nm peak may, however, be related to longer wavelength fluorophores.

As a consequence of ET, fluorescent emissions shift to longer wavelengths. The greater the amount of ET, the greater the shift to longer wavelength emissions at any one excitation wavelength. For example, when fluorescent intensity is plotted against emission wavelength for a 144 ppm Fluka humic acid solution excited at 330 nm, 360 nm and 390 nm, fluorescent intensity can be seen to increase quite significantly with increasing emission wavelength up to 520 nm (see Figure 4.21a).

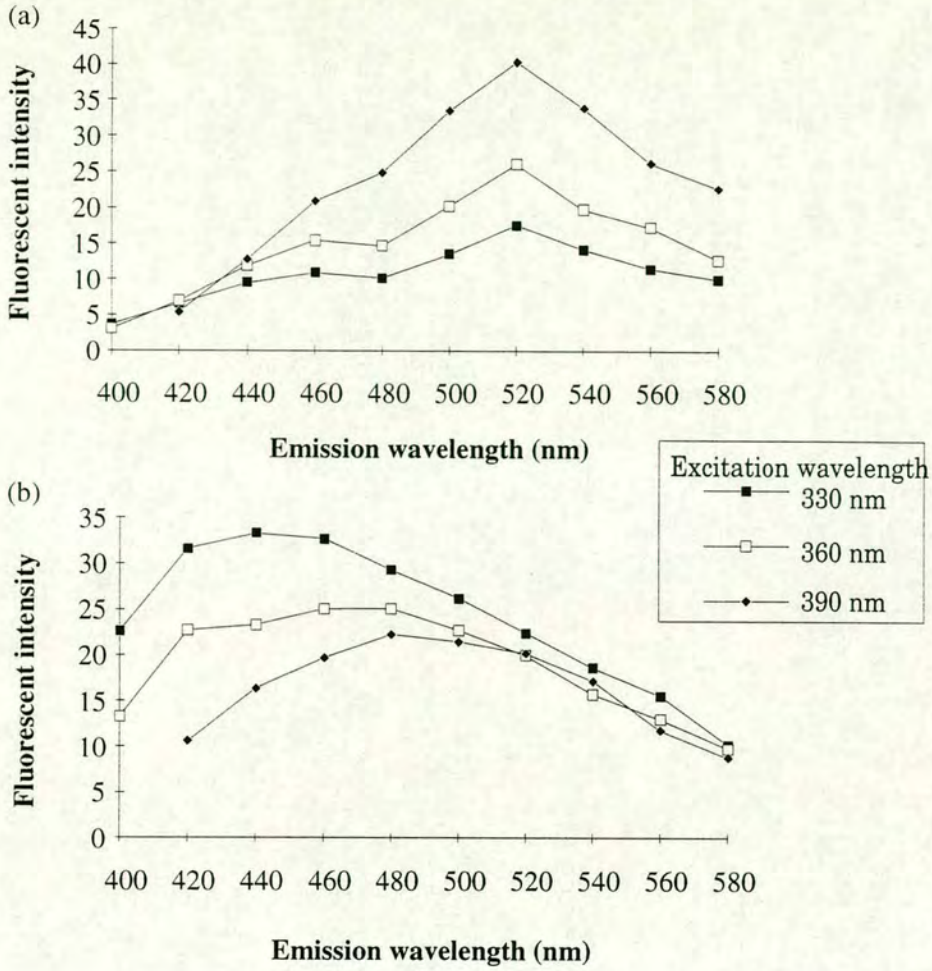

Figure 4.21 The effect concentration has on the wavelengths of fluorescence emitted. (a) 144 ppm Fluka humic acid solution showing long wavelength domination (b) 7 ppm Fluka humic acid solution showing short wavelength domination.

However, when fluorescent intensity was plotted against emission wavelength for a 7 ppm Fluka humic acid solution, fluorescence was dominated by shorter wavelength emissions (Figure 4.21b). Thus, while ET processes appear to be occurring in the 144 ppm humic acid solution, they are less significant in the 7 ppm humic acid solution. The fact that no colour change was detected at 470 nm with increasing concentration (see Figure 4.19, page 99) suggests that ET was not occurring in molecules of this group and implies that either their concentration was not sufficiently high or there were no molecules which absorb at wavelengths longer than 470-480 nm to transfer the energy to. Certainly no excitation peaks in coral or Fluka humic acid solutions were detected above 470 nm, although, as mentioned earlier (see section 4.3.2, pages 72-74), this does not necessarily mean that none were there.

Results in this section have suggested that it is possible to change the colour of Fluka humic acid fluorescence by increasing the concentration of all fluorophores equally (i.e. without changing their relative proportions). To test whether the same change in colour could be seen in coral solutions, EXEM spectra for 1M, 2.5M and 5M coral solutions, from a common source, were produced. Results showed that a slight shift towards the green was predicted with increasing concentration at each excitation wavelength (see Figure 4.22).

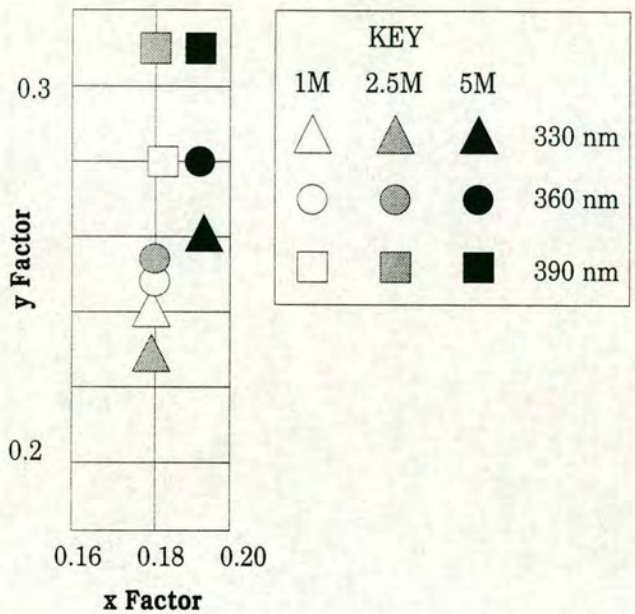

Figure 4.22 1M, 2.5M and 5M coral solutions prepared from *Porites lutea* collected from Porites Bay. 330 nm, 360 nm and 390 nm = excitation wavelengths. Refer to Figure 4.14 on page 88 to assign colours to these points.

Thus, it would appear that increasing the absolute concentration of all fluorophores equally in coral solutions without changing their relative proportions can also produce a change in the colour of fluorescence, albeit a very slight change. Therefore, if there is a difference in the colour of fluorescent bands in the solid state, variations in the concentration of the two dominant fluorophore types between bright and dull bands could conceivably be one way in which this difference could be produced, with bright bands having a higher concentration of fluorophores than dull bands. However, the concentration of fluorophores in most bright and dull band coral solutions was not sufficiently high in either band to produce a significant difference in colour between the two. Thus, the only real difference between 1-5 M bright and dull band coral solutions was one of intensity.

The effect concentration has on the intensity of coral fluorescence: Not only can ET result in a shift towards longer wavelength emissions in Fluka humic acid and coral solutions, it also affects fluorescent intensity. For example, the total fluorescent intensity (the sum of all emissions produced at one excitation wavelength) of Fluka humic acid solutions excited at 330 nm, 360 nm and 390 nm increased when the concentration of Fluka humic acid increased from 7 ppm to 36 ppm and then decreased with increasing concentration thereafter (see Figure 4.23).

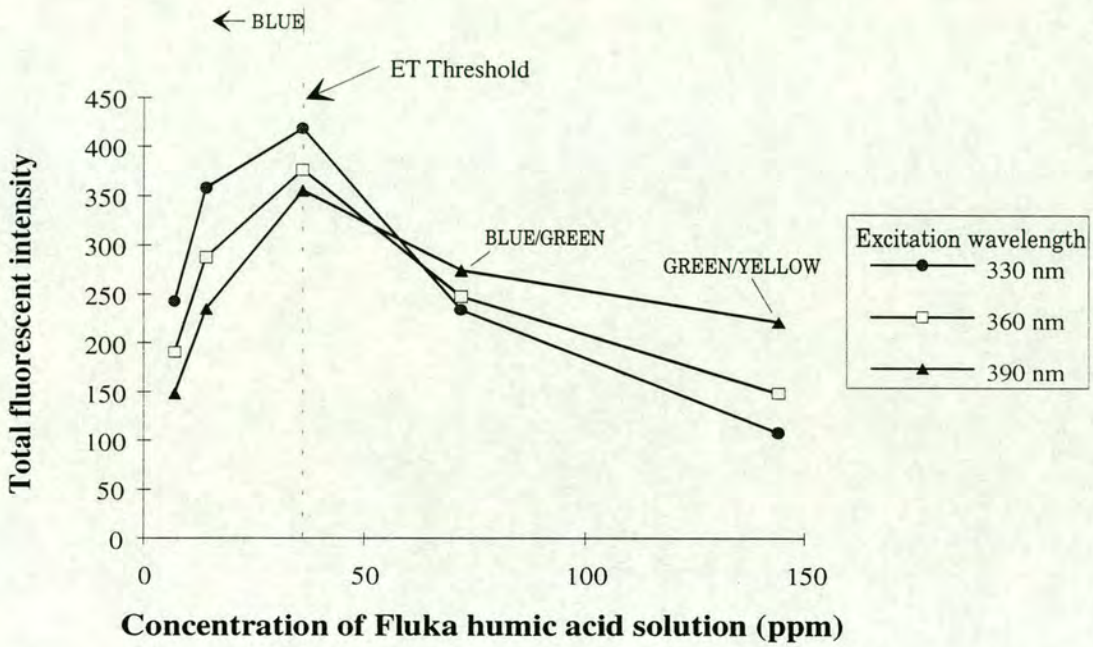

Figure 4.23 The total fluorescent intensity of Fluka humic acid solutions as a function of concentration. It can be seen that although total fluorescent intensity increases with increasing humic acid concentration up to 36 ppm, further increases in concentration result in a decrease in total fluorescent intensity.

Thus, dilution does not necessarily result in a decrease in total fluorescent intensity. One possible explanation to account for this is that as concentration decreases, ET processes become less effective and less energy is lost as a consequence. The point at which ET processes cease to be significant is referred to in this and subsequent chapters as the ET threshold. Below the ET threshold, changes in concentration do not result in a change in colour, merely a change in the intensity of fluorescent emissions. This is not to say that ET processes no longer occur below the ET threshold, just that its effects are too small to significantly affect colour (both visible and predicted). In order to determine the ET threshold point, the percentage change in total fluorescent intensity was calculated between the various humic acid solutions. When a 144 ppm Fluka humic acid solution was diluted to 72 ppm, a

uniform decrease in total fluorescent intensity at all excitation wavelengths was not seen (see Figure 4.24). In fact, total fluorescent intensity between 300 nm and 420 nm actually increased when this was done (also seen in Figure 4.23). However, when a 14 ppm Fluka humic acid solution was diluted to 7 ppm, a more or less uniform decrease in total fluorescent intensity for all excitation wavelengths was seen. These calculations suggested that while ET processes occurred above 72 ppm in humic acid solutions, they were no longer significant at concentrations less that ~14 ppm (i.e. the ET threshold for Fluka humic acid solutions was around 14 ppm. As this value is likely to be different for different fluorophores, it can not be assumed that the ET threshold in coral solutions will be similar, even if they do contain humic acids.

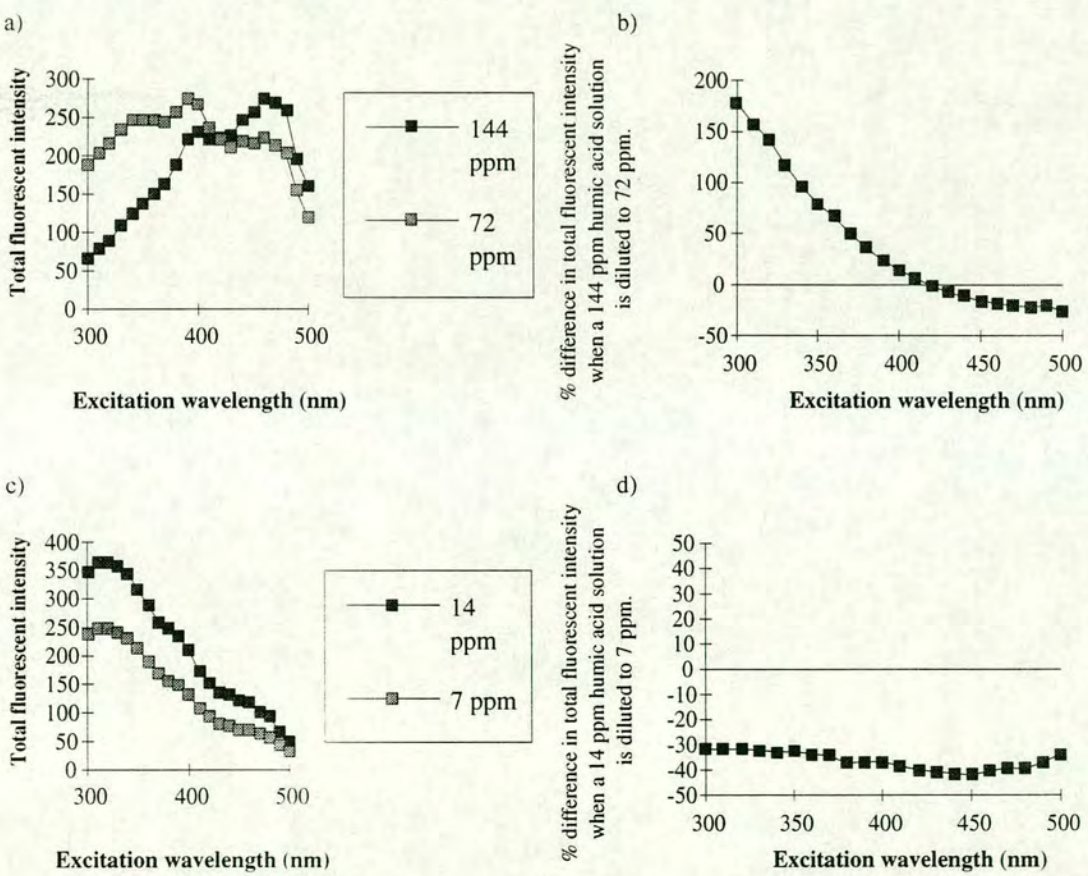

Figure 4.24 The effect dilution has on the total fluorescent intensity of Fluka humic acid.

For all 1M coral solutions analysed, diluting by 50% did not significantly alter the shape of the EXEM spectra, merely reduced the total fluorescent intensity (see Figure 4.25).

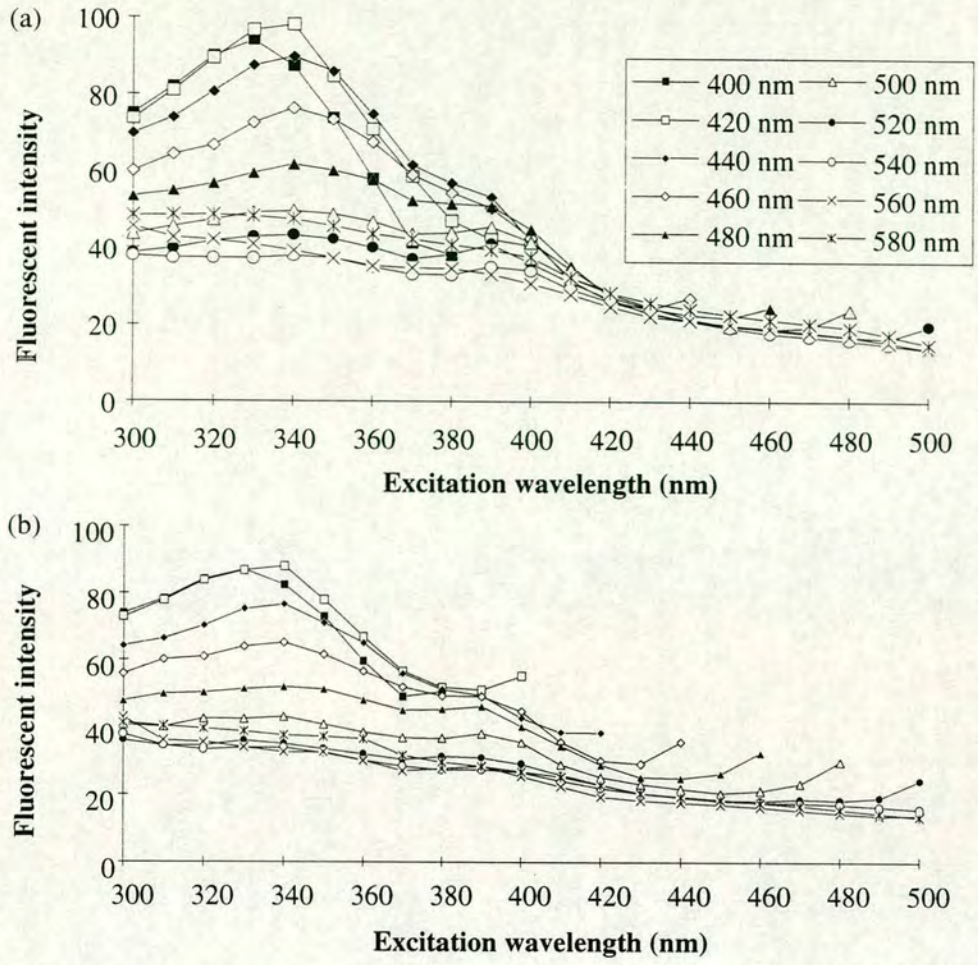

Figure 4.25 Diluting a 1M coral solution (PB-8-87) by 50%, (a) before and (b) after dilution.

Thus, it would appear that the concentration of fluorophores in most 1M coral solutions is below the ET threshold which is why it was possible to distinguish between bright and dull band coral solutions using fluorescent intensity. If the difference between bright and dull bands in the solid state is one of intensity, with bright bands being more intense than dull bands, concentration variations between the two band types could conceivably be one way in which this difference could be produced. In other words, bright bands have a higher concentration of fluorophores than dull bands and as a result fluoresce more intensely. However, this assumes that the concentration of fluorophores in the solid state is below the ET threshold (a topic which is discussed later in this section) and that solid state bright band fluorescent intensity is always greater than solid state dull band fluorescent intensity, a point which is discussed in Chapter 6.

The effect of absorbing impurities on Fluka and coral solution fluorescence: Although a change in the colour of coral solution fluorescence could be produced by increasing concentration, this change appeared to be very much quicker for humic acid solutions than coral solutions. This could simply be because the Fluka humic acid solutions contained a higher concentration of fluorophores. However, Fluka and Aldrich humic acid are also known to contain approximately 8.5% inorganic impurities (Susic *et al* 1991). These impurities were removed using XAD-2 resin which has a high affinity for humic acids. The use of XAD resins to concentrate humic acids is discussed in more detail in section 4.7 (see pages 123-132). When a 100 ppm Aldrich humic acid solution was passed through XAD-2 resin, the fluorescent intensity of the extract was much greater than the original solution[2]. Whilst the intensity of the 470 nm excitation peak remained essentially the same, there was an eight fold increase in the intensity of the 330-345 nm excitation peak which was now three times as intense as the 470 nm peak. The material not adsorbed to the resin passed into the residue phase and was visibly more coloured than the extract. This material, once adjusted to the same pH and concentration as the original solution, was found to have about twice the absorbence at 300 nm but only one fifth the fluorescent intensity as the original. Thus, it appeared that a large quantity of material which absorbed strongly in the short excitation wavelength region of the 100 ppm humic acid EXEM spectrum (brown coloured material) had been removed by the XAD-2 resin. These impurities could affect fluorescence in two ways: (1) by absorbing the exciting light and thus preventing the fluorophores from fluorescing (inner filter effects), and/or (2) by absorbing the fluorescence emitted by the fluorophores (radiative energy transfer). The former will result in a decrease in the intensity of all emission wavelengths equally which would not affect the colour of fluorescence. As far as the latter is concerned, the fact that impurities appeared to absorb shorter wavelengths to a greater extent than longer wavelengths could affect the colour of fluorescence as more blue light would be absorbed than green light. Thus, the more impurities of this nature there are, the higher the ratio of green to blue light. In addition to this, if the impurities are more soluble than the humic acids, as the concentration of both increased, the impurities would remain in solution longer and thus result in an even greater absorption of short wavelength emissions. The question then arises, "could the changes in the colour of Fluka humic acid fluorescence seen with increasing concentration be due to inner filter effects alone?" In order to test this, a 72 ppm Fluka humic acid solution was passed through

[2] This was done as part of an associated collaborative project with Matthews and myself, the results of which are in prep.

XAD-2 resin to remove the absorbing impurities. As discussed in section 4.7.1, page 124, XAD-2 resin is not 100% efficient and therefore the concentration of the extract is unlikely to be the same as the original solution (i.e. it will be lower). As it was not possible to determine the concentration of the extract, it will be referred to as X. The extract solution was adjusted to pH 9 before being divided into three equal parts, 'a', 'b' and 'c'. Part 'a' was unaltered, part 'b' was evaporated in an oven at 50°C such that its volume decreased by 50% thus producing a humic acid solution with concentration 2X, and part 'c' was diluted with DH_2O to produce a humic acid solution with concentration ¼X. Results show that ET does occur (see Figure 4.26).

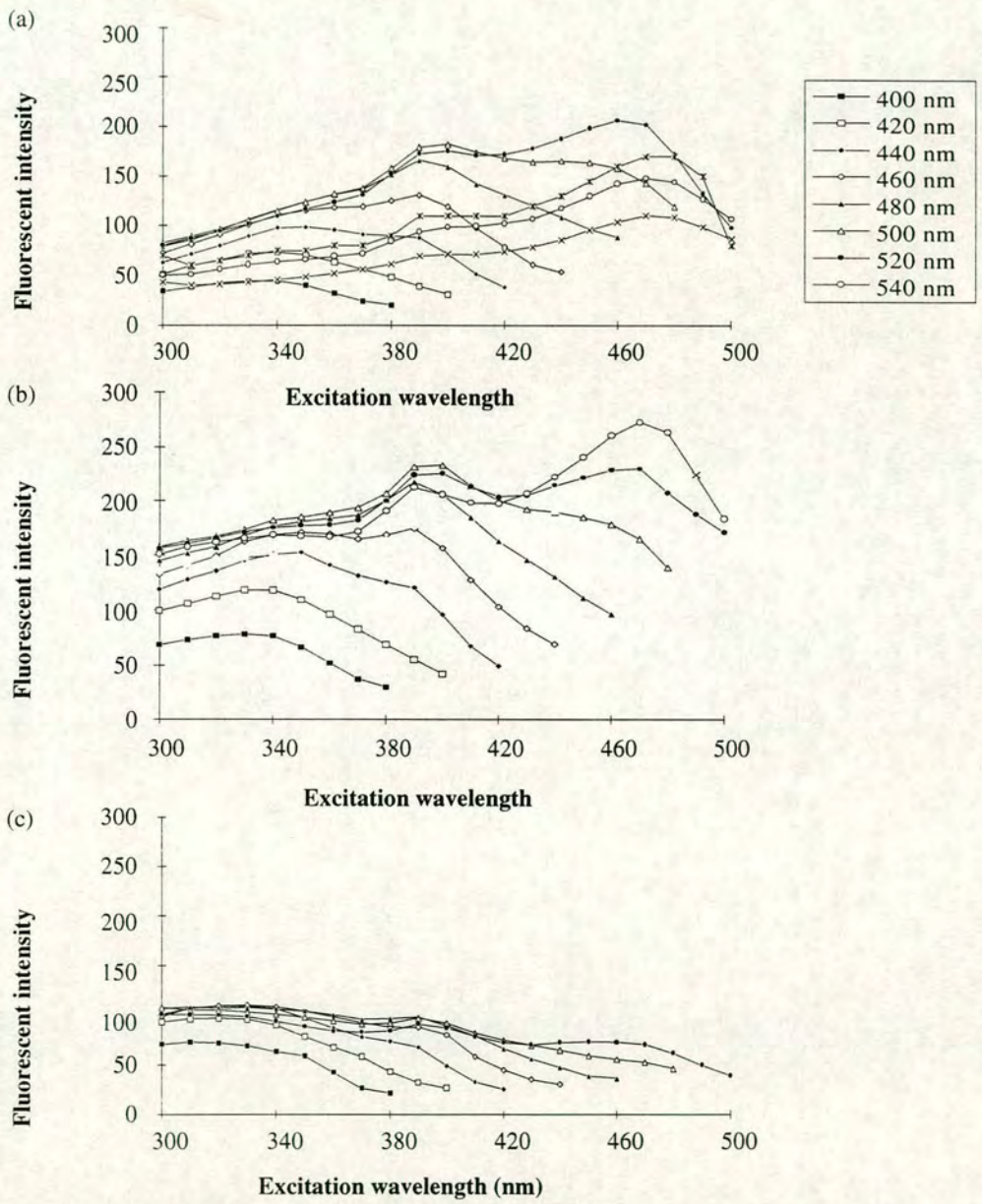

Figure 4.26 The effect that XAD-2 resin has on the fluorescence of Fluka humic acid with decreasing concentration. (a) 2X ppm (b) X ppm (c) ¼X ppm.

In Figure 4.27, the predicted colour of fluorescent emissions at 330 nm, 360 nm, 390 nm and 470 nm are shown for a 144 ppm Fluka humic acid solution, and the 2X humic acid extract. A 144 ppm humic acid solution is shown here because doubling the concentration of the original solution (72 ppm to 144 ppm) should mean that the concentration of fluorophores in the 2X extract more closely approximates that in the 144 ppm humic acid solution. Results show that at each excitation wavelength examined, the fluorescence emitted by the 144 ppm humic acid solution plus impurities is at longer wavelengths than the humic acid solution without the impurities. Despite the fact that the concentration of fluorophores in the 2X humic acid solution is slightly lower than in the 144 ppm humic acid solution, results nevertheless suggest that the impurities shift fluorescent emissions to longer wavelengths (see Figure 4.27).

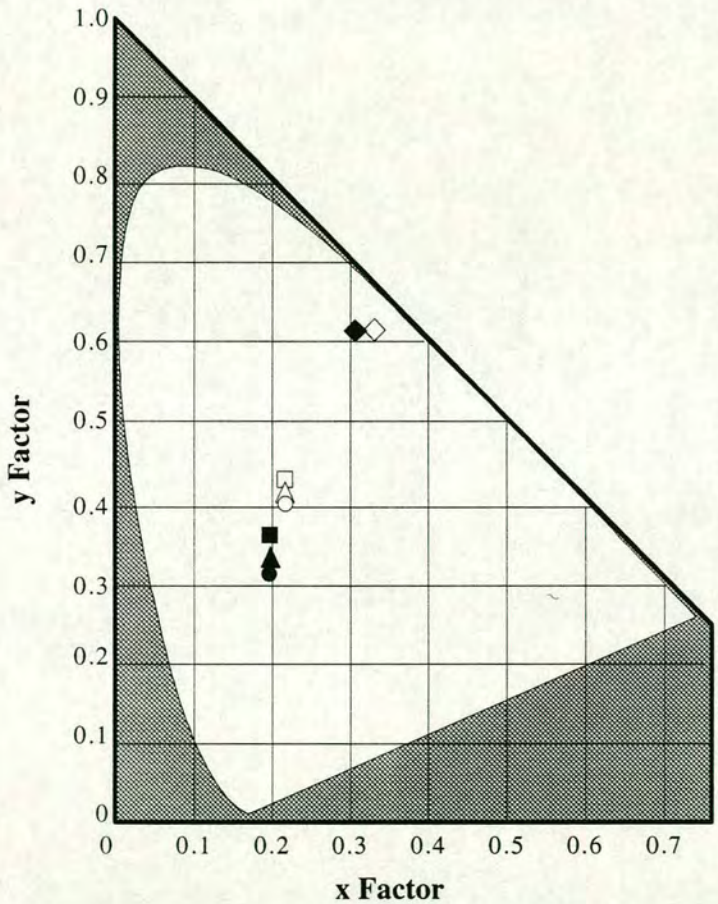

Figure 4.27 The predicted colour of fluorescent emissions at 330 nm, 360 nm, 390 nm and 470 nm for a 144 ppm Fluka humic acid solution with impurities and a humic acid solution of similar concentration which has had the impurities removed by being passed over the XAD-2 resin. Key: black = 2X ppm Fluka humic acid that has passed over XAD-2 resin; white = 144 ppm Fluka humic acid that has not passed over XAD-2 resin, circles = excitation at 330 nm, triangles = excitation at 360 nm, squares = excitation at 390 nm, diamonds = excitation at 470 nm. Refer to Figure 4.14 on page 88 to assign colours to these points.

"How do humic acid impurities affect fluorescence?" Results show that the 144 ppm humic acid solution that had not passed over the XAD-2 resin has a much lower total fluorescent intensity than the 2X humic acid solution (see Figure 4.11 on page 85 for 144 ppm Fluka humic acid solution than had not passed over XAD-2 resin). Absorption spectra for these two solutions showed that the 144 ppm solution absorbed much more between 300 nm and 360 nm compared to the 2X humic acid sample that had passed over XAD-2 resin (see Matthews *et al* in review). Thus, it would appear that the rapid change in colour seen when the concentration of Fluka humic acid was increased was brought about, in part at least, by the presence of impurities that preferentially absorbed short wavelengths.

Can Aldrich humic acid be used as a standard for quantifying coral fluorescence? Susic *et al* (1991) estimated the concentration of humic acids in corals by direct comparison with Aldrich humic acid. They did this by exciting humic acid solutions at 340 nm and measuring fluorescent emissions above 418 nm using a high pressure liquid chromatography system. They claimed that humic acids were well resolved from many other polar and acidic compounds producing a single sharp peak and that humic acid fluorescence was linear between 3.43 ppm and 42 ppm. Although work in this chapter has also suggested that humic acid concentration and fluorescent intensity were linearly related at low concentrations (<~14 ppm), this was not the case when concentration increased above this. As concentration increases above the ET threshold, total fluorescent intensity actually begins to decrease. At the concentrations studied in this chapter, only the intensity of the 490 nm humic acid peak increased fairly regularly with increasing concentration. However, this peak was not present in coral solutions or in the solid state (see later) and therefore can not be used as a standard for estimating coral humic acid concentration.

Therefore, although Fluka humic acid fluorescent intensity can be used to estimate the concentration of humic acid in dilute coral solutions, such estimates are unlikely to be very reliable for the following reasons:

(a) Coral fluorescence has been shown to be due to a number of fluorophores, not all of which are humic (Susic *et al* 1991). Therefore, comparing coral fluorescence to humic acid fluorescence will not necessarily give you a very accurate estimation of coral humic acid concentration.

(b) Fluka humic acid contains impurities which have been shown to effect EXEM spectra. Thus, the presence of unknown quantities of such impurities in humic acid solutions would make comparisons with coral solutions unreliable. Although these impurities could be removed using XAD-2 resin, the use of this resin in fluorescent studies is not recommended (see section 4.7, pages 123-132).

(c) Even when XAD-2 resin was used to remove the impurities from the Fluka humic acid solutions, fluorescent intensity and humic acid concentration were not linearly related. In other words, fluorescent intensity increased with increasing concentration below the ET threshold but decreased with increasing concentration above the ET threshold.

Bearing this in mind, the concentration of humic acid in 1M coral solutions was estimated by direct comparison with industrial humic acid EXEM spectra by comparing: (1) the intensity, and (2) the shape of coral and humic acid EXEM spectra. Comparisons suggested that the 14 ppm humic acid EXEM spectra most closely resembled typical 1M coral EXEM spectra (see Figure 4.28). The differences between the two probably reflect the problems associated with this comparative technique and the fact that industrial humic acid solutions appear to contain one extra fluorophore group at 470 nm.

Assuming the concentration of humic acid in typical coral solutions is 14 ppm, the concentration in solid coral skeletons can be estimated as follows. A 1M coral solution is the equivalent of 100 g in one litre. One litre of water occupies a volume of 1000 cm^3 whereas 100 g of coral skeleton occupies a volume of between 34 cm^3 and 100 cm^3 (depending on whether you take the density of aragonite which is 2.94 g/cm^3 or the skeletal bulk density which is approximately 1 g/cm^3). Therefore, in the solid state, the concentration of fluorophores in either band will be between 10 and 30 times greater than in the 1M coral solutions, i.e. between 140 ppm and 420 ppm. Although these estimates are higher than those reported by Susic *et al* (1991) who calculated the concentration of humic acid in coral skeletons to be approximately 56 ppm, it should be remembered that their estimates were for *Porites lutea* collected on the Great Barrier Reef and that location could be crucial.

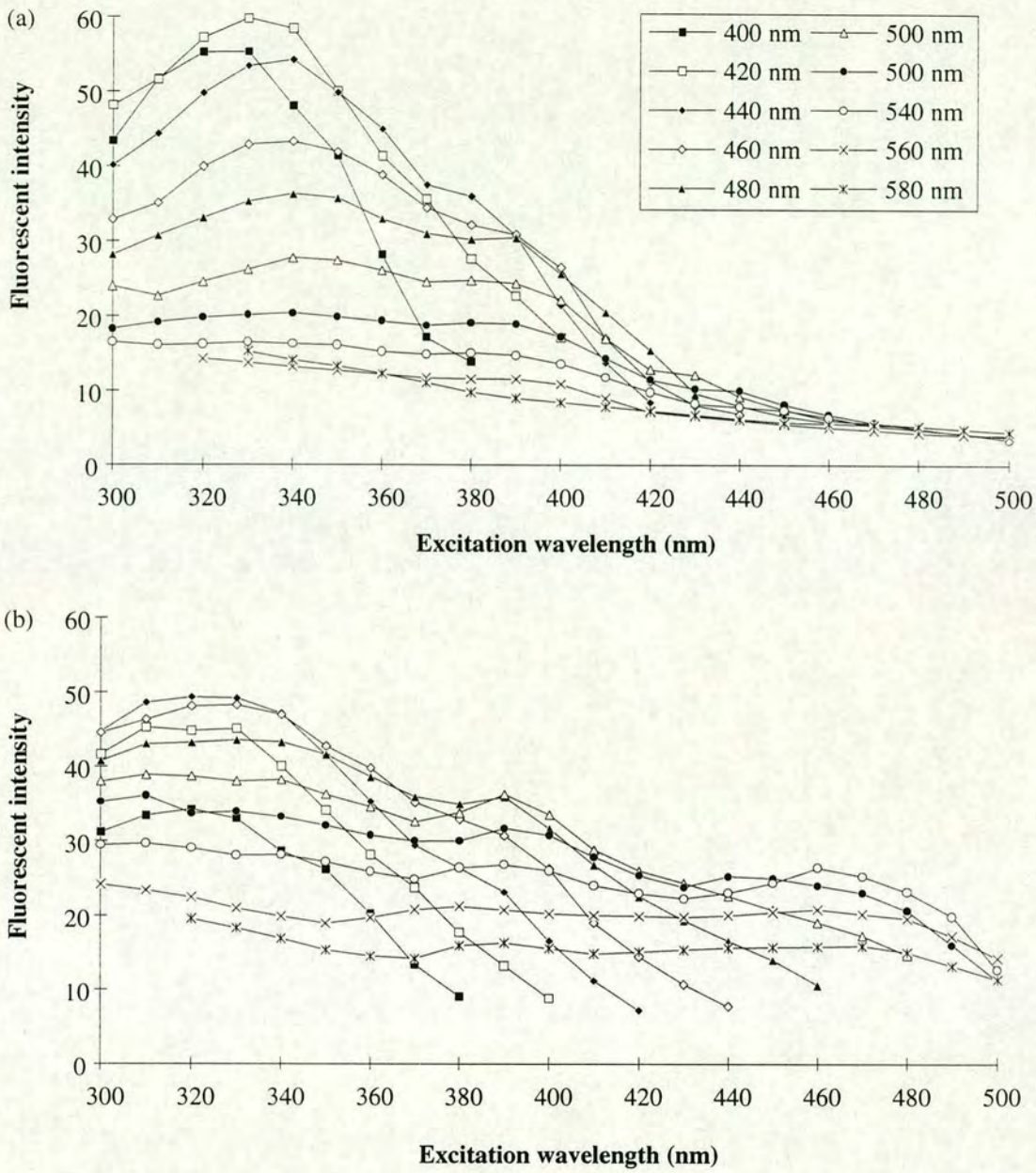

Figure 4.28 A typical 1M coral solution versus a low concentration Fluka humic acid solution. (a) a typical 1M coral solution, (b) 14 ppm Fluka humic acid solution.

Implications: The use of fluorescent intensity as a guide to fluorophore concentration: Results in this section suggest that fluorescent intensity could only be used as a means of estimating fluorophore concentration (in solution phase or the solid state) if the concentration was below the ET threshold. Although this appears to be the case in coral solutions, calculations suggest that the concentration of humic acids in the coral skeleton (both bright and dull bands) is between 140 ppm and 420 ppm. If these values are a true representation of the concentration of humic acids in

the coral skeleton, then by analogy with Fluka humic acid solutions, the ET threshold should have been surpassed and long wavelength colours should dominate fluorescent emissions for both bright and dull bands. If this was the case, fluorescent intensity should not be used as a means of estimating fluorophore concentration in solid coral skeletons.

The estimates of fluorophore concentration in the solid state are not, however, supported by visual observations (see Chapter 1, Figure 1.1, page 1, in which fluorescence in the Thai corals would appear to be dominated by short wavelengths). Assuming that the estimate of fluorophore concentration is the correct order of magnitude and that the ET threshold in both bands has been surpassed, a blue fluorescence should not, in theory, be seen (i.e. a longer wavelength colour would be expected such as green or yellow). However, even allowing for the fact that reflected/scattered light from the u/v lamp may make the coral appear bluer than it is, dull bands are definitely blue and not any other colour. This would suggest that in dull bands at least, fluorophore concentration is below the ET threshold. This may even be true of bright bands as it is far from clear whether they are in fact a different colour to dull bands. Thus, unless my estimate of fluorophore concentration in *Porites lutea* from Ko Phuket is inaccurate (and there is some reason to suppose that it could be), it may be the manner in which the fluorophores are associated in the coral skeleton that is the key to fluorescent banding. This subject forms the basis of Chapter 5. Using coral fluorescent intensity as a tool for hindcasting some, as yet unidentified, environmental factor is discussed in Chapter 6.

Migrating excitation peaks: It can be seen that at a fixed concentration, the excitation wavelength maxima between 330-345 nm varies according to the emission wavelength examined. For example, in AQ-25-87 BB (see Figure 4.29), the excitation maxima for individual emission wavelengths are as follows: 330 nm for the 400 nm emission wavelength, 335 nm for the 420 nm emission wavelength, 340 nm for the 440 nm emission wavelength, 345 nm for the 460 nm emission wavelength and 350 nm for the 480 nm emission wavelength.

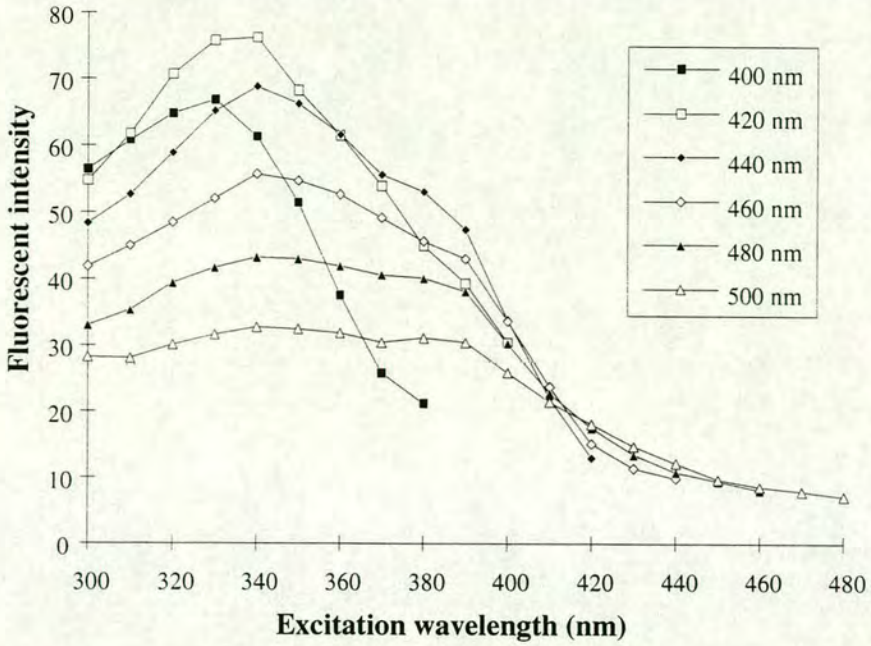

Figure 4.29 The migration of excitation peaks that occurs at a fixed concentration (AQ-25-87 BB).

Although the reason(s) for this were not absolutely identified, the following factors may be involved:

(a) When a sample is excited at a fixed wavelength, the emissions produced are always at longer wavelengths (lower energy). As a result of this, there will be an interval immediately after the excitation wavelength in which only a very small amount of fluorescence is emitted. As the interval between excitation and emission wavelength increases, the amount of fluorescence emitted increases up to a point (excitation maximum) and then decreases thereafter. For example, in Figure 4.29, the amount of fluorescence emitted at 400 nm can be seen to increase as excitation wavelength decreases from 380 nm to 330 nm and then decrease thereafter. Thus, the decrease in the amount of fluorescence emitted at 400 nm when excited between 330 nm and 380 nm is due to the fact that the interval between excitation and emission wavelength is decreasing and longer wavelength fluorescent emissions become more dominant. Obviously the shortest emission wavelength is going to be the first effected by this which is why the 400 nm excitation wavelength maximum is at 330 nm while the 460 nm excitation wavelength maximum is at 345 nm. However, such an explanation does apply to the 390 nm excitation peak as this peak did not migrate in any of the EXEM spectra taken. Thus some other explanation may be required.

(b) Migration could also be due to inner filter quenching in which impurities absorb more at shorter wavelengths. However, XRD work has suggested that the coral skeleton used in this chapter was almost pure aragonite (see Chapter 3, section 3.3, page 55).

(c) The broad 330-345 nm excitation peak could be due to the existence of numerous smaller excitation peaks where as the narrower 390 nm excitation peak could be due to a single type of fluorophore. Continued work in this field would, however, have to be carried out to confirm this.

The effects of concentration: A summary:

(1) The colour of fluorescent emissions in Aldrich and Fluka humic acid solutions is controlled by: (1) ET processes at concentrations greater than approximately 14 ppm, and (2) the presence of (brown) impurities which absorb short wavelengths preferentially and thus have a greater effect on the 330-345 nm excitation peak than the 390 nm excitation peak. Their presence is also part of the reason why a change in the colour of fluorescent emissions was seen so rapidly with increasing industrial humic acid concentration. Although ET may influence the colour of fluorescence in very concentrated coral solutions, the concentration of fluorophores in 1M coral solutions was below this level and as a result, no significant changes in colour were seen.

(2) Increasing concentration can also affect fluorescent intensity. Below the ET threshold, increasing fluorophore concentration results in an increase in florescent intensity whereas above the ET threshold, it results in a decrease in fluorescent intensity. In the solid state, the concentration of fluorophores was calculated to be well above the ET threshold in which case fluorescent intensity should not be compared with environmental records. However, the fact that these calculations are not supported by visual observations could suggest that it is the manner in which coral fluorophores are associated in the skeleton that is the important factor.

(3) Finally, results in this section have shown that fluorescent banding can be produced by increasing the absolute concentration of all fluorophores in a mixture without changing their relative proportions, rather than requiring the input of a specific fluorophore type as proposed by Boto and Isdale (1985).

4.6.2 The effect of the metal ions iron and manganese on the fluorescence of humic acid and coral solutions: The effects of iron and manganese on the luminescent properties of carbonates have been well documented (see Miller 1988 for a full review). For example, cathodoluminescent studies, which involve the emission of light resulting from the bombardment of a surface with electrons or cathode rays, have shown that manganese is the primary luminescent activator and iron is the primary luminescent quencher in calcite (Sommer 1972; Frank *et al* 1982; Miller 1988). However, little work seems to have been done on the effect of these ions on coral fluorescence. Although Boto and Isdale (1985) have demonstrated that iron quenches humic acid fluorescence, EXEM spectral analysis will allow the effect of metal ions on coral fluorescence to be studied in greater detail.

How metal ions could be associated with the coral skeleton: Metals other than Ca^{2+} can be associated with the coral skeleton in a number of ways.

(a) **By substitution for Ca^{2+}**: Fe^{2+}, Mn^{2+} and Mg^{2+} commonly substitute for Ca^{2+} in calcite whereas Sr^{2+}, Pb^{2+} and Ba^{2+} commonly substitute for Ca^{2+} in aragonite. Thus, it is very unlikely that Fe^{2+}, Fe^{3+} and Mn^{2+} would substitute for Ca^{2+} in *Porites lutea* as their skeleton is made of aragonite. This probably explains why the concentrations of iron and manganese are low in corals of the family *Poritidae* (0-15 ppm Mn^{2+} and 0-210 ppm Fe^{2+}) whereas Sr^{2+} concentrations are very high (0.71-1.03%). All estimates of ion concentrations in coral skeletons were taken from Howard and Brown (1984).

(b) **Metals can also interact with organic molecules**: These interactions can be classified into two groups. In the first group, the interaction is predominantly ionic (i.e. electron donating/accepting) and generally involves the alkali metals (Li, Na, K, Rb, Cs) and the alkali earth metals (Be, Mg, Ca, Sr, Ba, Ra). These metals typically react with acidic organic groups such as carboxylic acids and alcohols, and result in the formation of ionic salts. In addition to this, these metals also form co-ordination compounds (linkage of two atoms by a pair of electrons both of which are provided by one of the atoms, the ligand) in which ligands provide an electron pair to bond to a metal atom. Some of these ligands are highly selective for a particular ion even in the presence of other chemically similar ions. The second group consists of metals with a lesser tendency to form ionic bonds and includes all the remaining metallic elements including the transition elements and the heavy metals. Bonding is either covalent, in which

case the metal and counter ion each provide an electron to bond formation, or co-ordinate in which the ligand provides both electrons. In principle, any organic molecule containing trivalent nitrogen or phosphorous, divalent oxygen or sulphur, can act as a ligand. Since humic acids are believed to contain carboxylic and alcohol functional groups as well as nitrogenous compounds (Thurman *et al* 1988), a large number of metal-humic complexes are possible. Further evidence to suggest that metal ions are associated with humic acids was provided by Larson and Hufnal (1980) who suggested that the formation of humic acids was catalysed by Mn^{2+} and Fe^{3+}.

(c) Metal ions can also be adsorbed onto the coral surface or bound into clays (Deer *et al* 1966).

Method and materials: The effect of iron and manganese on the fluorescence of Fluka humic acid was examined using iron (II) chloride tetrahydrate ($FeCl_2$) and manganese chloride ($MnCl_2$) supplied by the Aldrich Chemical Company. Even though Fe^{2+} is the most stable state of iron in both acid and alkaline solutions, mild oxidants such as oxygen are capable of oxidising iron (II) to iron (III) and consequently the more stable state of iron in the presence of both air and water is Fe^{3+}. Therefore, would the $FeCl_2$ oxidise to $FeCl_3$? In acidic solutions, the conversion of Fe^{2+} to Fe^{3+} is slow (due to kinetic factors), but in alkaline or neutral conditions the oxidation is rapid. As all solutions were adjusted to pH 9, iron was probably in the Fe^{3+} state. Iron is expressed as $Fe^{2+/3}$ since the relative abundance's of each ion were unknown. A number of solutions were made up in which the concentration of Fluka humic acid was kept constant at 14 ppm while the concentration of $Fe^{2+/3+}$ and Mn^{2+} was as follows: 10 ppm, 20 ppm, 50 ppm, 100 ppm and 200 ppm.

Results and discussion:

The effect iron has on the fluorescent characteristics of humic acid solutions: The addition of 10 ppm $Fe^{2+/3+}$ to the Fluka humic acid solution resulted in a significant decrease in fluorescence (see Figure 4.30) and complete absorption of fluorescence was achieved when 100 ppm of $Fe^{2+/3+}$ was added. Similar decreases in fluorescent intensity were obtained when $Fe^{2+/3+}$ was added to 1M coral solutions. These results were attributed to strong iron absorption/quenching. It is interesting to note that the 330-345 nm excitation peak group appeared to be more affected by the

presence of iron than fluorescent emissions produced when the solutions were excited at longer wavelengths.

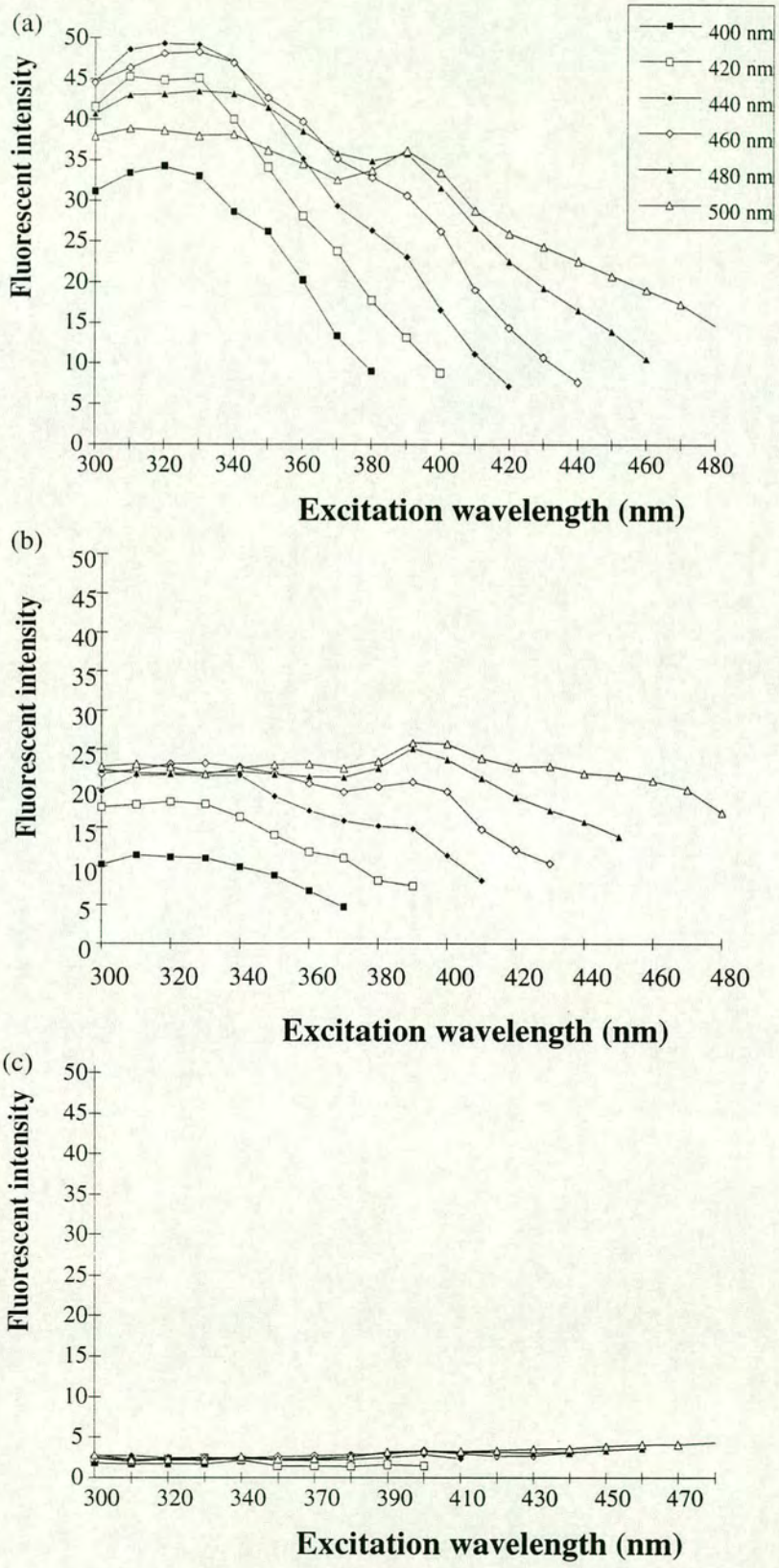

Figure 4.30 The effect of $Fe^{2+/3+}$ on the fluorescence of a 14 ppm Fluka Humic solution. (a) 14 ppm Fluka humic acid solution without iron. (b) 14 ppm Fluka humic acid solution with 10 ppm $Fe^{2+/3+}$(c) 14 ppm Fluka humic acid solution with 100 ppm $Fe^{2+/3+}$.

One possible explanation to account for this could be connected with the observation that iron chloride solutions absorb proportionally more radiation at shorter wavelengths (see Figure 4.31).

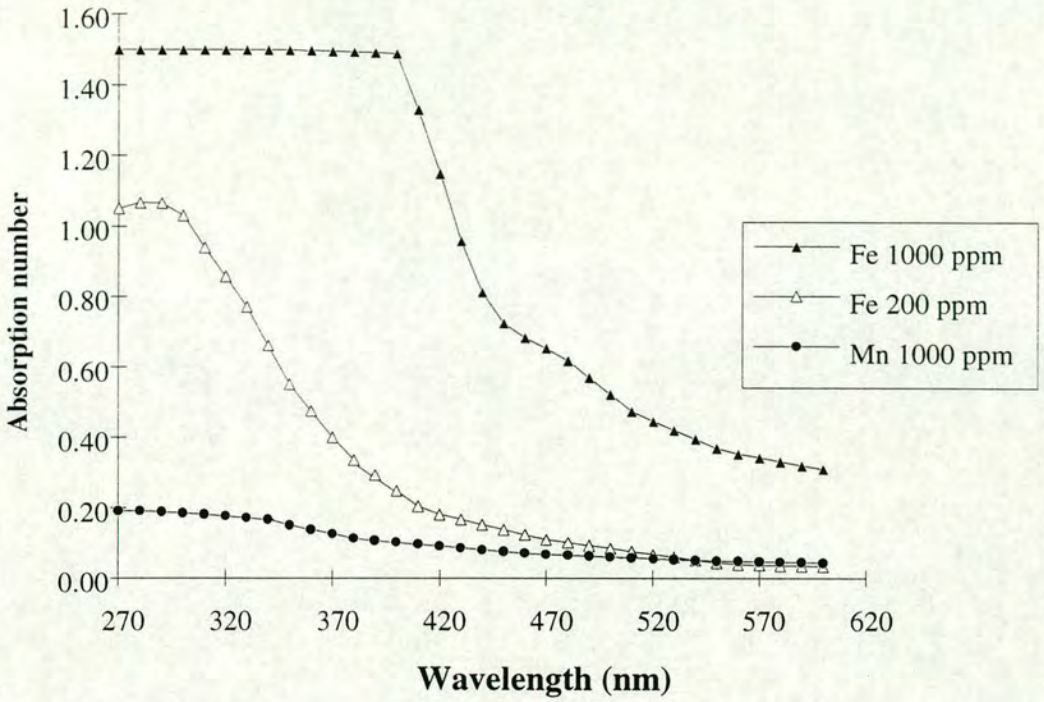

Figure 4.31 Absorption spectra of iron and manganese. Because of the limitation of the absorption machine used, absorption could only be measured up to 1.5 absorption units (97% absorption).

As a consequence of this, the 330-345 nm excitation peak was quenched to a greater degree than the 390 nm excitation peak. Therefore, the addition of small amounts of iron could, as a consequence of absorption, affect the colour of fluorescence in the same way as the inorganic impurities in Fluka humic acid do, i.e. by enhancing longer wavelength emissions. As the concentration of iron in corals of the family *Poritidae* has been estimated at between 0 and 210 ppm (Howard and Brown 1984), iron could have a significant effect on both the colour and intensity of coral fluorescence. However, the extent of its influence depends not only on its concentration in the skeleton, but also on its relationship to the fluorophores. In the solution phase, the iron is free to interact with the fluorophores and thus absorb both the incoming exciting light and the outgoing fluorescence. In the solid state, however, the iron may not be directly associated with the fluorophores (i.e. part of the same molecule) and thus, it may not necessarily have the same effect as in the solution phase. The effect iron has on coral fluorescence in the solid state was investigated during the first field trip. A solution of $FeCl_2$ was made up and sealed

within a plastic container. It was not possible to determine the concentration of iron in the bag throughout the experiment because of the unknown extent of seawater dilution. At the reef front, a plastic bag was opened and allowed to fill with seawater. The plastic container containing the iron solution was inserted into this bag which was then placed around a living coral head and secured with an elastic band. The plastic container was then opened and the bag was shaken to ensure the iron solution was well mixed with the seawater. The bag was left on the coral head for 4 hours after which it was removed. The coral head was then tagged and left for a year before collection. When this coral was cut in half an iron band could be clearly seen. When excited under a u/v lamp, this band could be seen to quench coral fluorescence (see Figure 4.32).

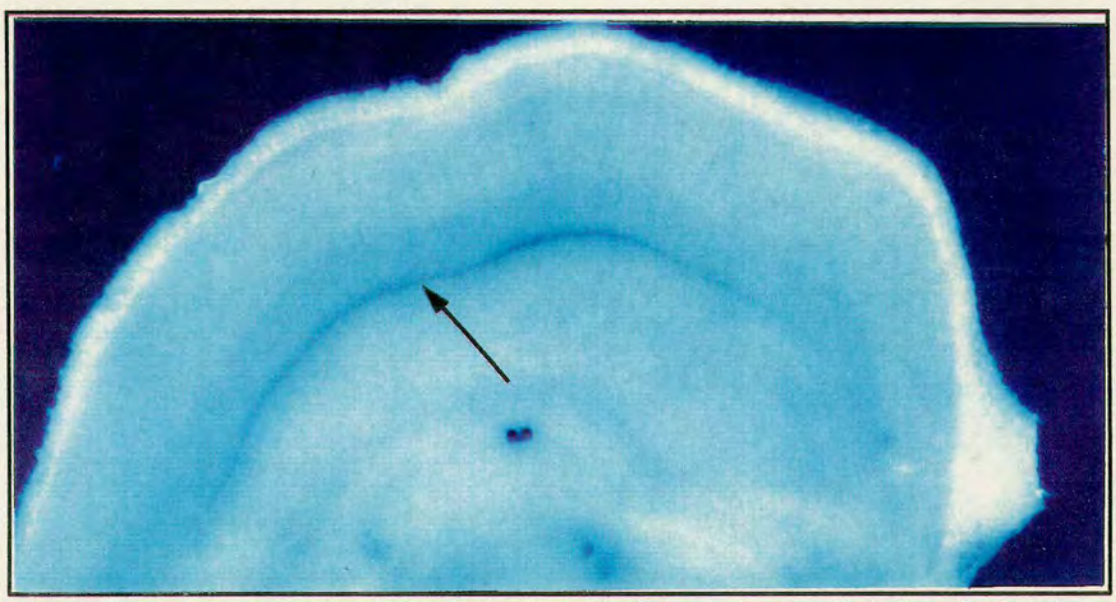

Figure 4.32 Staining a living coral head with iron chloride. The iron band is indicated arrowed.

As mentioned before, iron in either oxidation state is unlikely to substitute for Ca^{2+} in *Porites lutea*. For this reason, Brown *et al* (1991) have suggested that the iron band they saw in some of the *Porites lutea* from Tin Smelter Reef was due to Fe^{3+} that precipitated on exposed coral skeleton as a consequence of tissue retraction brought about by abnormally high iron concentrations in the surrounding seawater. A similar explanation could account for the appearance of an iron band (seen in Figure 4.32) when the coral was spiked with iron (II) chloride. Although the iron in this case is not directly associated with the coral fluorophores, it reduces coral fluorescence by absorbing the emitted fluorescence. These results suggest that the presence of iron, associated with coral fluorophores directly or indirectly, can inhibit/quench coral fluorescence.

The effect manganese has on the fluorescent characteristics of humic acid solutions: Although manganese has been described as a fluorescent enhancer in cathodoluminescent studies, results in this section suggest that it quenches (i.e. suppresses) both solution phase (see Figure 4.33) and solid state (see Figure 4.34) humic acid fluorescence in a similar was to iron (i.e. preferential absorption of short wavelengths).

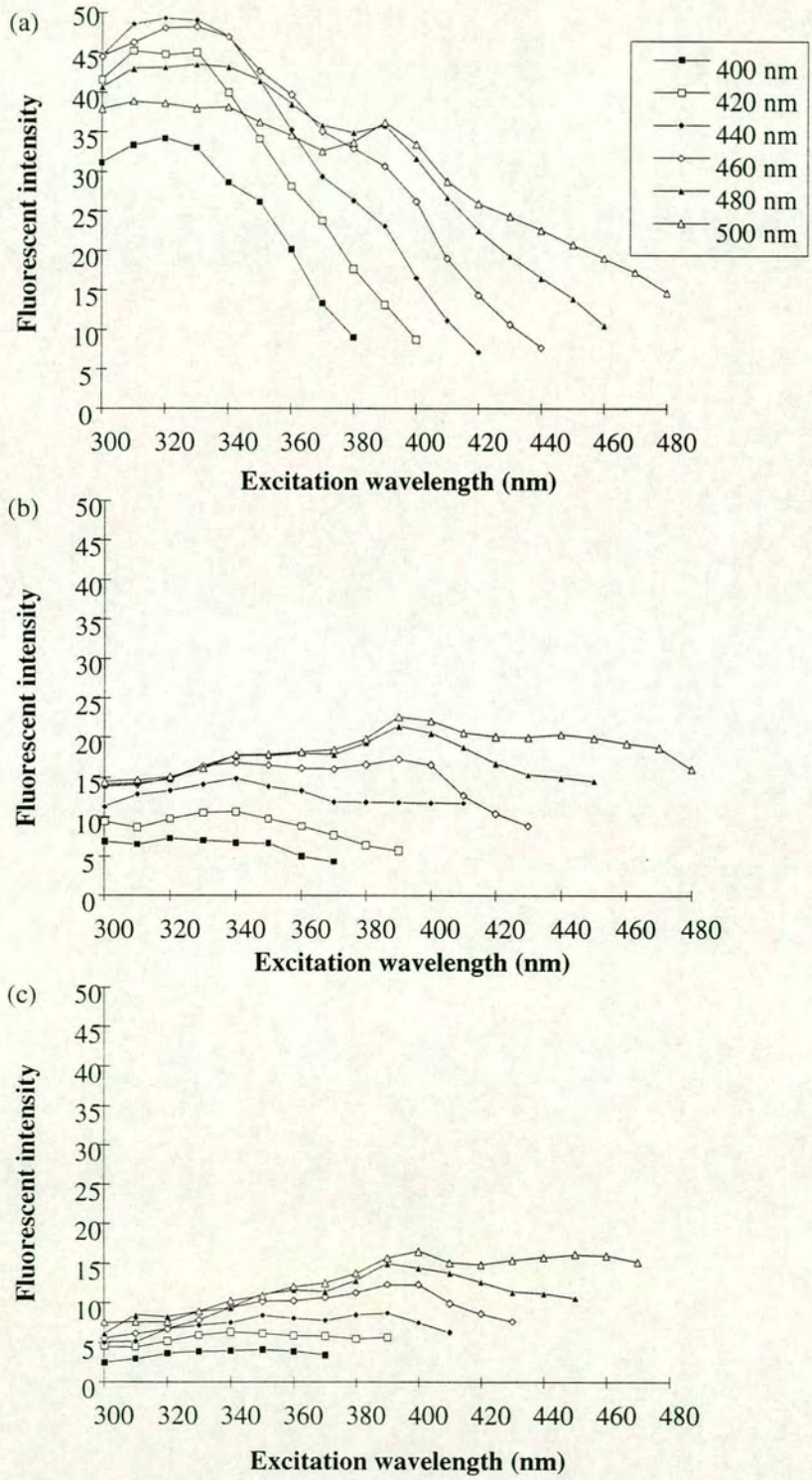

Figure 4.33 The effect of manganese on the fluorescence of a 14 ppm Fluka Humic solution. (a) 14 ppm Fluka humic acid solution without manganese. (b) 14 ppm Fluka humic acid solution with 20 ppm Mn^{2+}. (c) 14 ppm Fluka humic acid solution with 100 ppm Mn^{2+}.

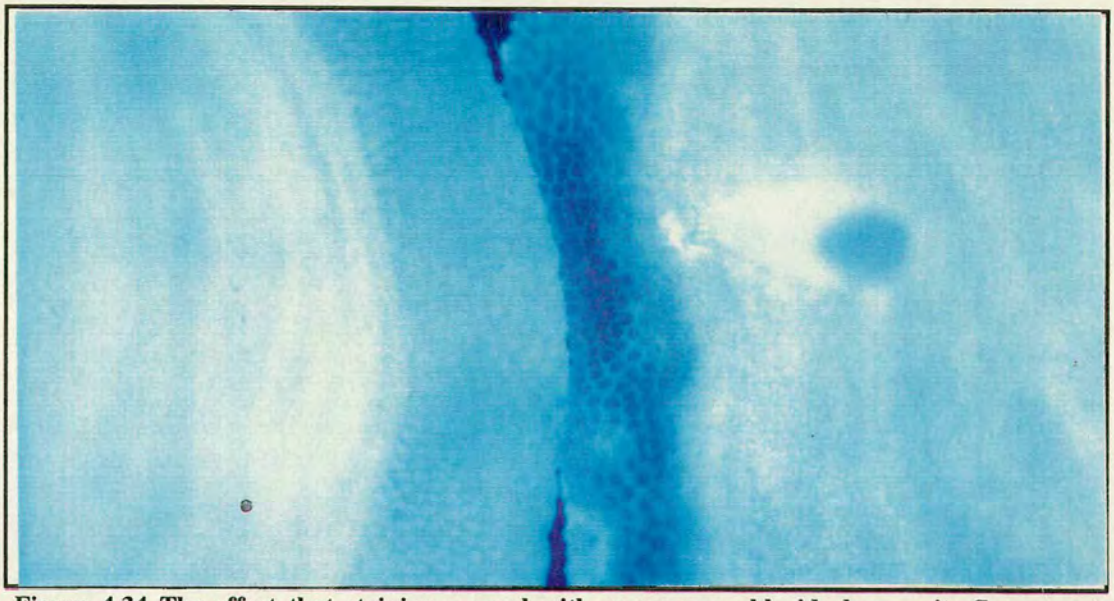

Figure 4.34 The effect that staining a coral with manganese chloride has on its fluorescent properties. The outer surface of the coral on the right hand side was stained with manganese chloride whilst the left hand side was unstained.

It is interesting to note that a much higher concentration of manganese is required to produce the same degree of quenching as iron (i.e. a 10 ppm iron solution has the same effect as a 20 ppm manganese solution on a 14 ppm humic acid fluorescence). The reason why manganese is a less effective fluorescent quencher is probably due to the fact that it absorbs less radiation over the range 270-580 nm (see Figure 4.31, page 118).

Although manganese has the potential to reduce coral fluorescence, its concentration in corals of the family *Poritidae* (0 and 15 ppm) would appear to be too low to have a significant effect on coral fluorescence.

The effects of iron and manganese: A summary: Although the concentration of manganese found in coral skeletons of the family *Poritidae* would appear to be too low to significantly affect coral fluorescence, the presence of iron at the concentrations commonly found in corals of this family could significantly affect coral fluorescence (i.e. fluorescent intensity) and possibly even cause fluorescent banding. However, if iron was to cause fluorescent banding in *Porites lutea*, one might expect its concentrations to vary seasonally (i.e. between bright and dull bands for example). As no compositional variations were discovered using secondary ion mass spectrometry (Allison 1994), it therefore seems unlikely that iron is the main cause of fluorescent banding.

4.6.3 Summary/Key points:

(1) Concentration certainly does have a significant affect on coral fluorescence both in terms of colour and intensity. Although variations in the concentration of fluorophores in coral solutions can result in a change in the colour of fluorescence, a large dilution factor was involved in the making of coral solutions which is why a change in colour with increasing concentration was not seen. Although increasing the concentration of fluorophores above the ET results in a reduction in fluorescent intensity, at the concentrations examined in this study (all below the ET threshold), fluorescent intensity can be used as a means of distinguishing bright and dull bands (the former being more fluorescent than the latter). Thus, it is conceivable that fluorescent banding is due to variations in the concentration of the same or very similar types of fluorophores. However, although calculations suggest that the concentration of fluorophores in the solid state is above the ET threshold in both bright and dull bands, visual observations do not support this. Thus, some other mechanism must also be involved in fluorescent banding.

(2) Results have shown that both manganese and iron can quench coral fluorescence. Although there would not appear to be enough manganese in coral skeletons to have a significant effect, iron is present in sufficiently high concentrations to affect fluorescence. However, no seasonal variations in iron content were found between bright and dull bands (Allison 1994) and on this basis it would appear that the presence of iron in the coral skeleton is not the major control on fluorescent banding.

4.7 EXEM SPECTRA OF CORAL EXTRACTS OBTAINED USING XAD-2 RESIN:

XAD-2 resin has been used extensively in the past as a means of extracting humic acids from very dilute solutions such as seawater. As the fluorescent signature of seawater samples was to be investigated in this study, the use of XAD-2 resin was explored. It was hoped that such work would determine whether XAD-2 resin can be used as an efficient way of concentrating coral fluorophores.

4.7.1 Background:

The analytical separation of humic compounds from solutions has usually been carried out by methods involving adsorption. Many workers have favoured inorganic adsorbents such as activated carbon, silica gel, magnesia and calcium carbonate. However, recoveries with these tend to be low, partly because of the inefficiency of the adsorption stage, but also because of the difficulty in eluting the humic acids (i.e. recovering the humics once they have been separated). Other methods of extraction include ion exchange, freeze drying or roto-evaporation. Although anion exchange resins, such as Duolite A-7, have a high capacity for anionic organic solutes, they also concentrate inorganic anions present in the water. Freeze drying may alter the humic acid structure as -H from a hydroxyl group and -OH from a carboxyl group are removed to form water. This reduces water solubility and may lead to a structural change. Various authors have shown that higher recoveries of humic acids can be achieved by the use of micro-porous polystyrene beads such as Amberlite XAD resins (Stuermer and Harvey 1974) which is why XAD resin was used in this section.

4.7.2 Methods and materials:

Choosing the appropriate XAD resin: Most work involving the extraction of humic acids from soils, seawater an even coral skeletons, has involved the use of XAD-2 resin (Mantoura and Riley 1975, Stuermer and Harvey 1977, Fu and Pocklington 1983, Boto and Isdale 1985, Lara *et al* 1993). One of the most important problems encountered when using XAD resins is a phenomenon known as resin bleed in which contaminants associated with the resin, such as chemical preservatives, get into the extract. One of the advantages of styrene-divinlybenzene XAD resins such as XAD-2, over acrylic-ester resins such as XAD-8 is that resin bleed is significantly lower in the former (Aiken 1988) which is why XAD-2 resin

was used in this study. Amberlite XAD-2 is a macro-reticular resin based on polystyrene. It has a porosity of 0.42 and an average specific surface area of $300m^2$ g^{-1}. It is produced as white insoluble beads supplied in a 20-60 mesh size range. There are, however, a number of problems that are specific to Amberlite XAD-2 resin:

(a) Fu and Pocklington (1983) recorded a drastic decrease in adsorption efficiency after 300 to 1000 bed volumes of seawater had passed through the column[3]. The efficiency thereafter remained more or less constant. They postulated that after a certain amount of organic material has been adsorbed, the resin becomes saturated. Only new material with a high affinity for the resin will be adsorbed at the expense of material with a lower affinity. Although the precise amount of coral solution added to the resin varied according to the amount of coral skeleton dissolved in each experiment (commonly between 40 mls and 200 mls), the amount added was never more than 300 bed volumes.

(b) Fu and Pocklington (1983) also note that although XAD-2 resin has been accepted as a useful adsorbent for organic matter, its total recovery of natural organic matter from seawater is low (at best *ca.* 40%).

(c) As high molecular mass humic acids have lower oxygen contents, are much less water soluble and more hydrophobic than low molecular mass humic acids, they are adsorbed onto the resin more effectively (Mantoura and Riley 1975). This means that the final isolate may not be representative of the original organic material in the coral skeleton.

Choosing the appropriate eluent: The earliest reagent used for extracting humic acids from soils and seawater samples was NaOH, and with minor modifications, it is still the most widely used today giving the largest yields[4]. However, because the chemicals used in the isolation procedure can interact with the humic acids, a number of chemical and physical alterations often occur. The main problems associated with the three most commonly used eluents are considered here:

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[3]The term 'bed volume' is taken to represent the volume of XAD-2 resin used in the column.

[4] Because humic acids are insoluble below pH 2 (a feature which has been attributed to the presence of a large number of acid functional groups), they are not extracted with acids.

(a) **Sodium hydroxide**: Humic acids are often extracted with a 0.1 N NaOH solution which has a pH of 13. At this pH, ester hydrolysis is very rapid. In addition to this, when NaOH is used, desalting procedures are required to remove sodium salts from the extracts. However, desalting procedures such as dialysis can result in the loss of low molecular mass material (Stuermer and Harvey 1974) whereas ion exchange resins can irreversibly adsorb organic compounds (Stuermer and Harvey 1977).

(b) **Methanol**: Methanolysis reactions can occur with both carboxylic acid functional groups and organic esters. However, as the esterfication of carboxylic acid functional groups is catalysed by acids and not bases, the problem can be avoided by extraction under alkaline conditions.

(c) **Ammonium hydroxide**: In addition to aminolysis reactions with organic esters in the presence of a base, $-NH_3$ can interact with other components in humic substances such as phenols. Evidence also suggests that the use of NH_4OH as an eluent often results in higher nitrogen contents in the final isolates (Stuermer and Harvey 1977).

In order to determine whether the common eluents NaOH, CH_4 and NH_4OH could be used in fluorescent studies, EXEM and absorption spectra were produced over the range 360-580 nm. The eluents did not absorb strongly over this range and were basically non-fluorescent. Thus, they should not affect humic acid fluorescence and as such, were all considered suitable for the purposes of this study. Following the work of Fu and Pocklington (1983) who demonstrated that higher humic acid recoveries were achieved when organic and alkaline eluents were combined rather than being used individually, a combination of CH_4 and NH_4OH was used as the eluent in this section. This combination of eluents also avoided the need to desalt the extracts.

Extraction procedure: As new XAD-2 resin contains many impurities some of which fluoresce, it was soxhlet-extracted with acetone following the method of Mantoura and Riley (1975). The resin was then evaporated to dryness in an oven at 50°C to remove any left over acetone. 3.5 g (dry mass) of soxhlet-extracted XAD-2 resin was then accurately weighted out and loaded into a gravity column. Glass wool plugs were then secured on top of the resin to prevent large particles from entering as well as stopping the XAD-2 resin from floating to the surface. It was essential to

have an equal amount of resin in each column if reliable comparisons between extracts from different columns were to be made. The more resin a column had, the greater its potential to adsorb humic acids. When soxhlet-extracted resin was rinsed with DH_2O, the rinse displayed two main excitation peaks, one at 280 nm and the other at 330-345 nm (see Figure 4.35). These peaks coincide with coral and tryptophan excitation peaks and could therefore be a source of confusion.

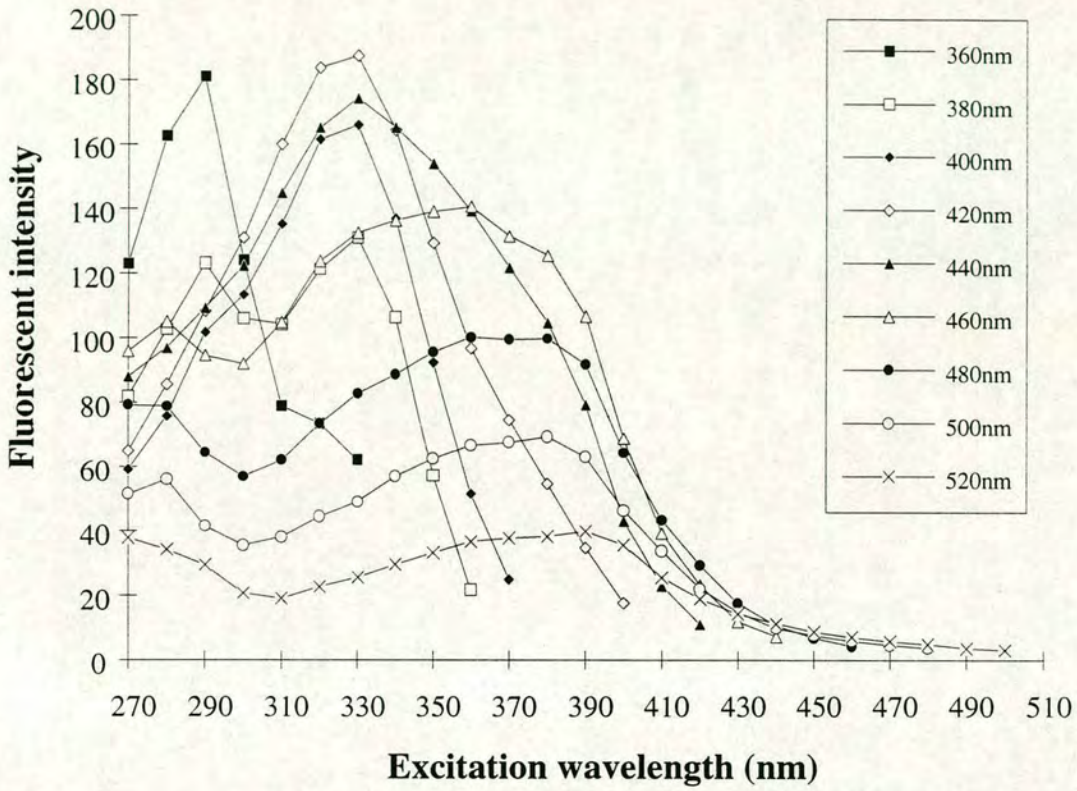


Figure 4.35 EXEM spectra of DH_2O after having passed over soxhlet-extracted resin.

As soxhlet extraction failed to remove all the impurities from the resin, further cleaning was required. The resin was first rinsed with 500 mls of DH_2O. 100 mls of 1M HCl was then added to remove any acid-soluble impurities from the resin. DH_2O (usually 500 mls) was then added until a negative test with silver nitrate was achieved. This occurred when all the chloride ions from the previous step were removed. 400 mls of a 50/50 mixture of 2N NH_4OH and CH_4 was added to remove any alkali-soluble impurities from the resin. The resin was then rinsed with 500 mls of DH_2O to remove the NH_4OH and CH_4. Although XAD-2 resin can extract organics over a wide pH range, the most efficient recovery of humic acids from 'garden' peat was achieved at pH 2.2 or below with only a *ca.* 2% increase in

adsorption efficiency when pH was lowered from 2.2 to 1 (Mantoura and Riley, 1975). Thus, the pH of the resin was adjusted by rinsing it with 100 mls of HCl adjusted to pH 2.2. It is also the reason why the pH of the coral solutions was adjusted to 2.2 (using HCl and NH_4OH) before being added to the column. Mantoura and Riley (1975) have reported that flow rate can influence the recovery of humic acids. They recommend a flow rate of less than 0.5 bed volumes per minute with maximum recovery being obtained when the eluent was allowed to stand in the column overnight. Although Stuermer and Harvey (1977) observed a marked decrease when flow rates were higher than 1 bed volume per minute, Fu and Pocklington (1983) showed that the decrease was insignificant below three bed volumes per minute. A flow rate of one bed volume per minute was used in this section. The residue (i.e. the solution containing substances that were not absorbed onto the resin) was passed through the column a total of three times in an attempt to maximise humic acid uptake. The humic acid was then eluted from the column with 400 mls of a 50/50 mixture of 2N NH_4OH and CH_4. In order to concentrate the fluorophores and remove NH_4OH and CH_4, both the residue and the extract were evaporated down to 20 mls in an oven at 50°C (the NH_4OH and CH_4 should vaporise) and the pH was adjusted to 9.

A summary of the technique:

(1) XAD resin was used to extract humic acids because of its high recovery rate relative to other techniques. XAD-2 resin was used because resin bleed was significantly lower compared to other XAD resins.

(2) NH_4OH and CH_4 were the chosen eluents for the following reasons: (1) they were non-fluorescent, (2) when used together they were more effective than when used individually, and (3) because potentially damaging desalting procedures were avoided.

(3) Because of impurities in the resin, it had to be soxhlet-extracted and rinsed a number of times before coral solutions, adjusted to pH 2.2, were added. The solution was passed through the column three times at a flow rate of 1 bed volume per minute before the humic acids were eluted.

(4) Both residue and extract phases were evaporated to 20 mls and the pH was adjusted to 9 before examination.

4.7.3 Results and discussion: Although a considerable amount of time was spent cleaning the resin, it was never completely free of contaminants (see Figure 4.36).

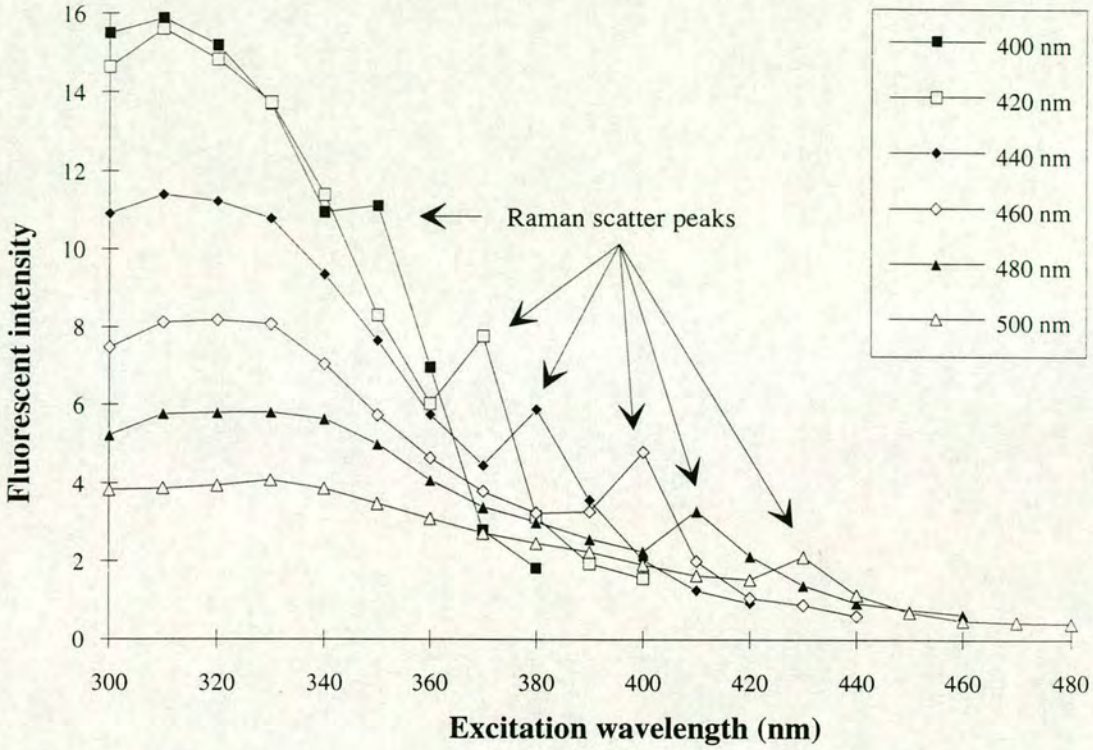


Figure 4.36 Blank EXEM spectrum. Emission wavelengths above 500 nm are not measured because the fluorescent intensity of the original data (i.e. the data not adjusted to PMT response) was too low to produce reliable data.

This would not have been such a problem if: (1) the fluorescent characteristics of blank[5] were significantly different from coral extracts, and (2) the blank EXEM spectra were similar between runs. Unfortunately blank EXEM spectra were similar to coral extract EXEM spectra (although considerably less intense). In addition, the intensity of blank EXEM spectra varied between runs which suggested that each column was contaminated to a different degree thus making it impossible to simply subtract a blank value from the extract.

Results also showed that the residue phase was often as fluorescent or even more fluorescent than the extract once it had been adjusted to pH 9 (see Figure 4.37).

[5] Blanks were produced when cleaned XAD-2 resin, which had not had a coral solution passed through it, was extracted with 400 mls of a 50/50 mixture of 2N NH_4OH and methanol and then evaporated down to 20 mls.

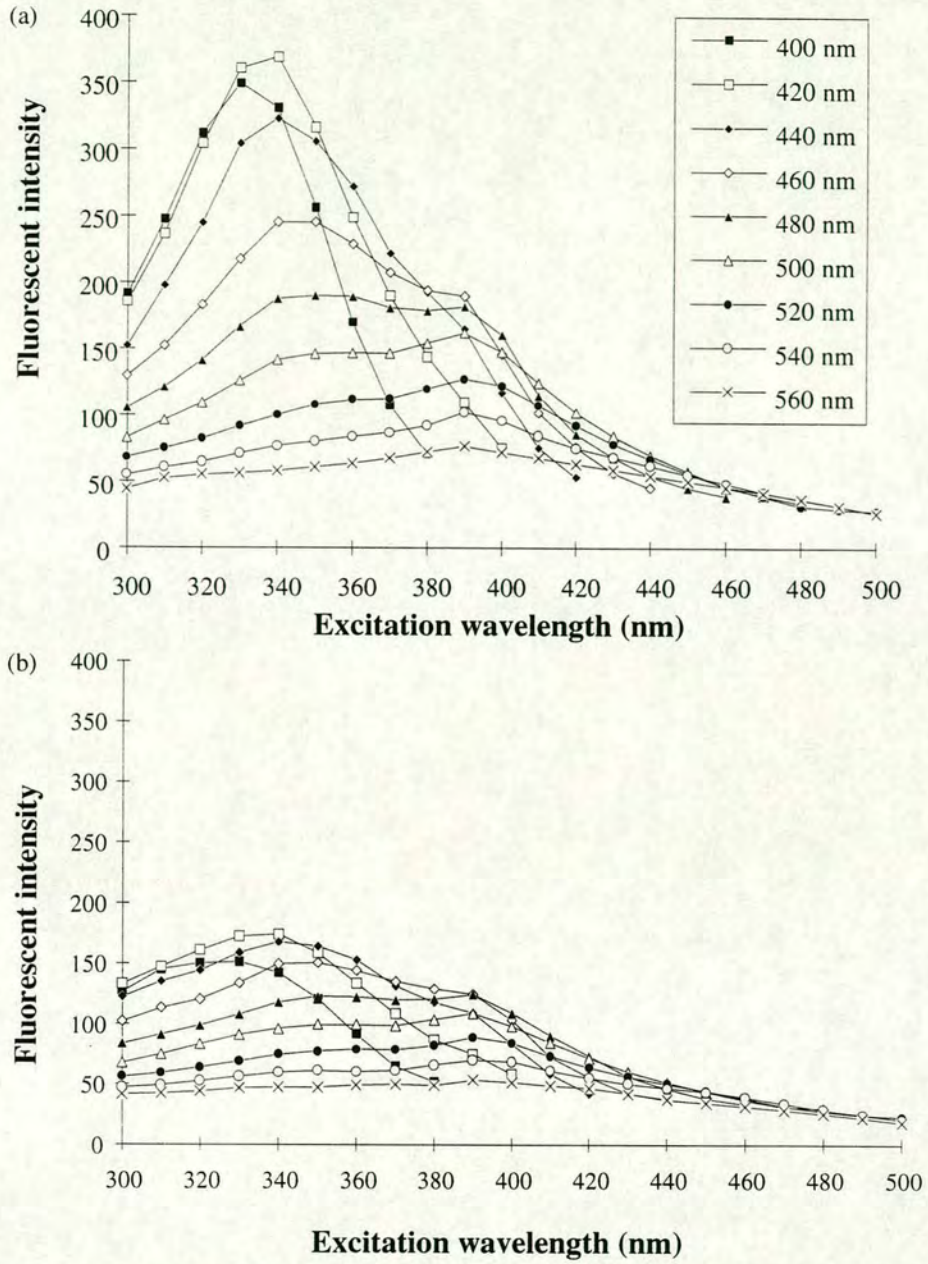


Figure 4.37 (a) Residue versus (b) extract.

The fact that the residue phase was often more fluorescent than the blank suggested that the majority of the residue phase fluorescence was due to the presence of coral fluorophores rather than to impurities from the resin. These fluorophores would undoubtedly contribute to coral fluorescence in the solid state and their exclusion from the extract phase suggests one or both of the following:

(a) The resin was unable to remove all the fluorophores from the coral solution, a factor that could be attributed to XAD-2 resin saturation (i.e. adsorption sites full) or poor extraction efficiency. In order to determine the extent of resin

efficiency/saturation, 2 g, 5 g and 10 g coral samples from a common source which had been crushed to 40 μm to homogenise the distribution of fluorophores, were each extracted in the manner described in section 4.7.2. Results showed that in both the extract and residue phases, the 10 g sample had a very prominent 390 nm excitation peak, the 2 g sample had a more prominent 330-345 nm excitation peak and the 5 g sample was in between. Although the shape and intensity of the EXEM spectra changed in both the extract and residue phases as sample mass increased, these variations can be explained in terms of concentration. As the sample mass increased, the number of fluorophores adsorbed onto the resin also increased thereby increasing the concentration of fluorophores in the extract phase. As the concentration of fluorophores in the extract phase increased, ET process became more significant and fluorescent emissions shifted to longer wavelengths. These results therefore suggest that the resin was unable to extract all the fluorophores (i.e. it was not 100% efficient, as suggested by Fu and Pocklington, 1983). Thus, the larger the number of fluorophores in the initial solution, the larger the number of fluorophores that would make their way into the residue phase. This would also explain why in the residue phase, fluorescent emissions appear to shift to longer wavelengths with increasing sample mass. If resin saturation was more significant, one might expect the EXEM spectra of the three extracts to be very similar, and the fact that they are not would suggest that resin efficiency is a more dominant factor.

(b) The resin selectively adsorbed certain fluorophores. Certainly, XAD-2 resin is known to have a high affinity for hydrophobic compounds such as humic acids and alcohols. Therefore, some of the fluorophores may not have been absorbed because they were associated with hydrophilic compounds. Although the residue and extract phases contained the same excitation peaks, this should not be used as evidence to suggest that the resin does not fractionate the coral fluorophores to a significant degree for the following reasons: (1) the concentration of non-humic/non-hydrophobic fluorophores could be very low, and/or (2) the fluorescence from these molecules could have been quenched by humic acid or something else and thus not seen. Although these fluorophores may not have a significant effect in solution where they are in direct competition for the available light with humic acids, their effect in the solid state may be different. The potential significance of this is discussed in Chapter 5.

Thus, in addition to the possibility of selective adsorption, XAD-2 resin would not appear to be very efficient in which case the final extract will provide potentially misleading information in terms of concentration and fluorophore type. Despite these problems, EXEM coral solution spectra were similar to those obtained for low concentration humic acid solutions (see Figure 4.38).

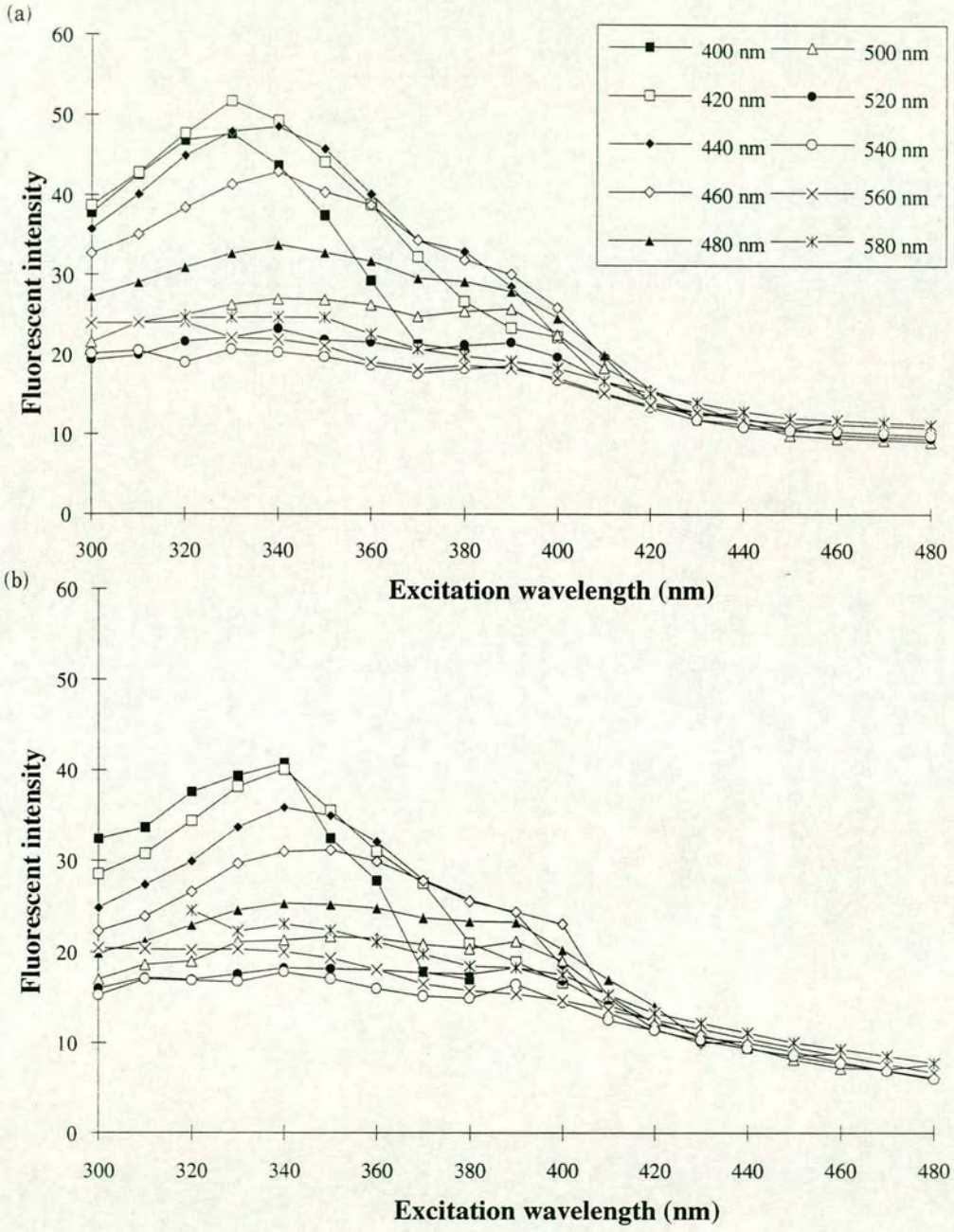


Figure 4.38 Bright (a) and dull (b) band extract EXEM spectra after XAD-2 extraction.

The dominance of the 330-345 nm excitation peak in both bright and dull band extract EXEM spectra suggested that the concentration of humic acids was below the ET threshold. Thus, as results also demonstrated that bright bands are more fluorescent than dull bands (also shown in Figure 4.38), it would appear that bright bands contain a higher concentration of humic acids than dull bands which is in keeping with the findings of Susic *et al* (1991) and those in section 4.4.2 on page 82.

4.7.4 Summary/ Key points:

(1) Results in this section have suggested that coral fluorescence should not be examined using XAD-2 resin. This is because truly representative samples containing all the essential components that contribute to coral fluorescence (i.e. non-humic fluorophores and impurities) that were free from resin contaminants were not obtained. In addition to this, as the isolation of coral fluorophores is likely to affect their fluorescent properties, an extraction procedure in which disruption is kept to a minimum is preferred.

(2) Although this technique has numerous problems, results in this section suggested that the concentration of humic acid in bright bands was greater than in dull bands thus confirming work by Susic *et al* (1991).

4.8 SOURCES OF FLUORESCENCE:

4.8.1 The *in situ* development of fluorophores: If coral fluorophores are developed *in situ*, then the most likely place for this to occur is in the polyp. When polyp tissue is examined under a u/v lamp, a bright orange fluorescence is noticed (see Figure 4.39). As results in this chapter have shown that increasing the concentration of humic acid from 14 ppm to 144 ppm results in a change in the colour of fluorescent emissions from blue to green/yellow, if the concentration was increased still further, even longer wavelength emissions would be expected (i.e. orange). Thus, it is not inconceivable that the polyp tissue represents a highly concentrated source of humic acids whose incorporation into the coral skeleton could influence coral fluorescence.

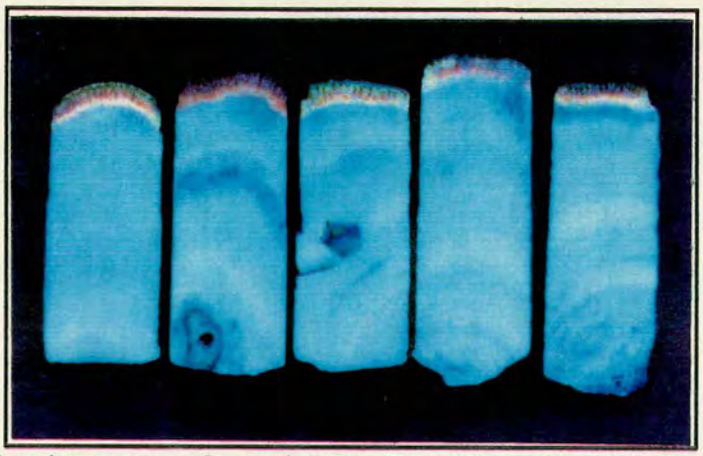


Figure 4.39 Polyp tissue seen under a u/v lamp. The polyp tissue can be seen at the living coral surface and is pink/orange in colour in this photograph for all the corals collected from Porites Bay in 1993 (PB-5-93).

Methods and materials: In order to study the fluorescent characteristics of polyp tissue, 2 g of unbleached coral skeleton containing polyp tissue was dissolved using 1N HCl. Care was taken not to include skeleton that did not contain polyp tissue as this would increase the amount of fluorophores normally associated with the skeleton. As the polyp tissue appeared to prevent carbonate dissolution, the sample was finely crushed before being dissolved. The solution was then evaporated to 20 mls and its pH adjusted to 9.

Results and discussion: Two excitation peaks were discovered in polyp tissue solutions, a dominant 390 nm excitation peak and a minor 330-345 nm excitation peak (see Figure 4.40). When this solution was diluted sufficiently, its EXEM spectrum closely resembled typical 1M coral solution EXEM spectra (i.e. 330-345 nm excitation peak dominating the 390 nm excitation peak). Thus, even though some of the fluorophores were probably derived from the skeleton, results suggested that polyp tissue contained fluorophores that were similar to those found in the skeleton although in much higher concentrations. In other words, the orange fluorescence seen when polyp tissue is examined under a u/v lamp could be due to the presence of a large amount of humic acid. It was not possible, however, to determine whether these humic acids were ingested from the marine environment or generated *in situ*.

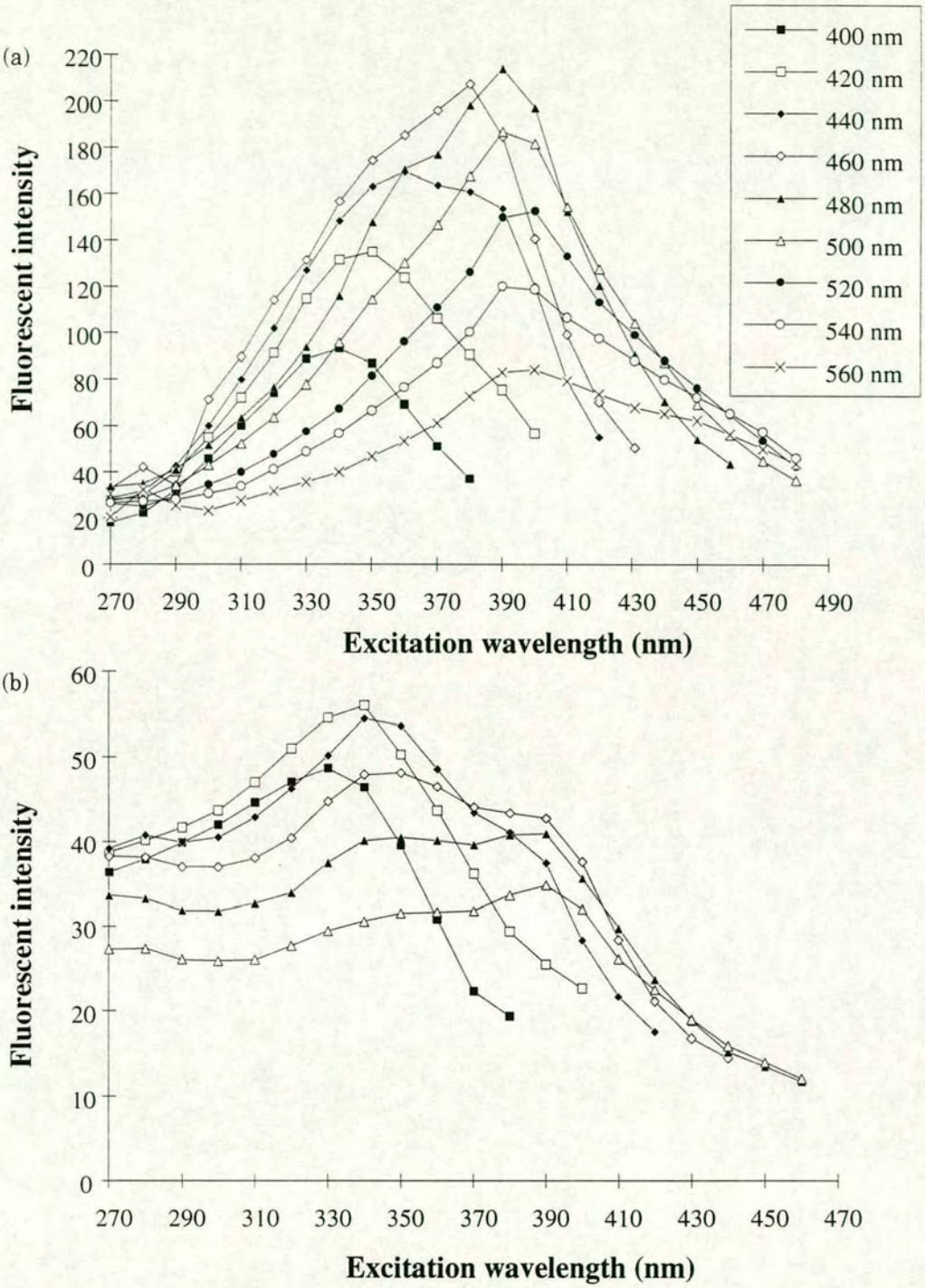


Figure 4.40 Polyp tissue EXEM spectra. (a) undiluted spectrum showing the 390 nm excitation peak, (b) diluted showing the 330-345 nm excitation peak.

Although humic acids are the dominant fluorophores in coral skeletons, Susic *et al* (1991) have suggested that 25-30% of coral fluorescence is due to other non-humic fluorophores. Because pteridines and flavins are the most abundant terminal catabolic products excreted by marine organisms, Dunlap and Susic (1985) have

suggested that these were the most likely fluorescent metabolites to be incorporated into coral skeletons. Isoxanthopterin, a common pteridine, and riboflavin, a common flavin, were therefore examined to see what contribution, if any, they could make to coral fluorescence. When an EXEM spectrum was produced for isoxanthopterin, two excitation peaks were seen, a dominant 340 nm excitation peak and a smaller 280-285 nm excitation peak (see Figure 4.41a).

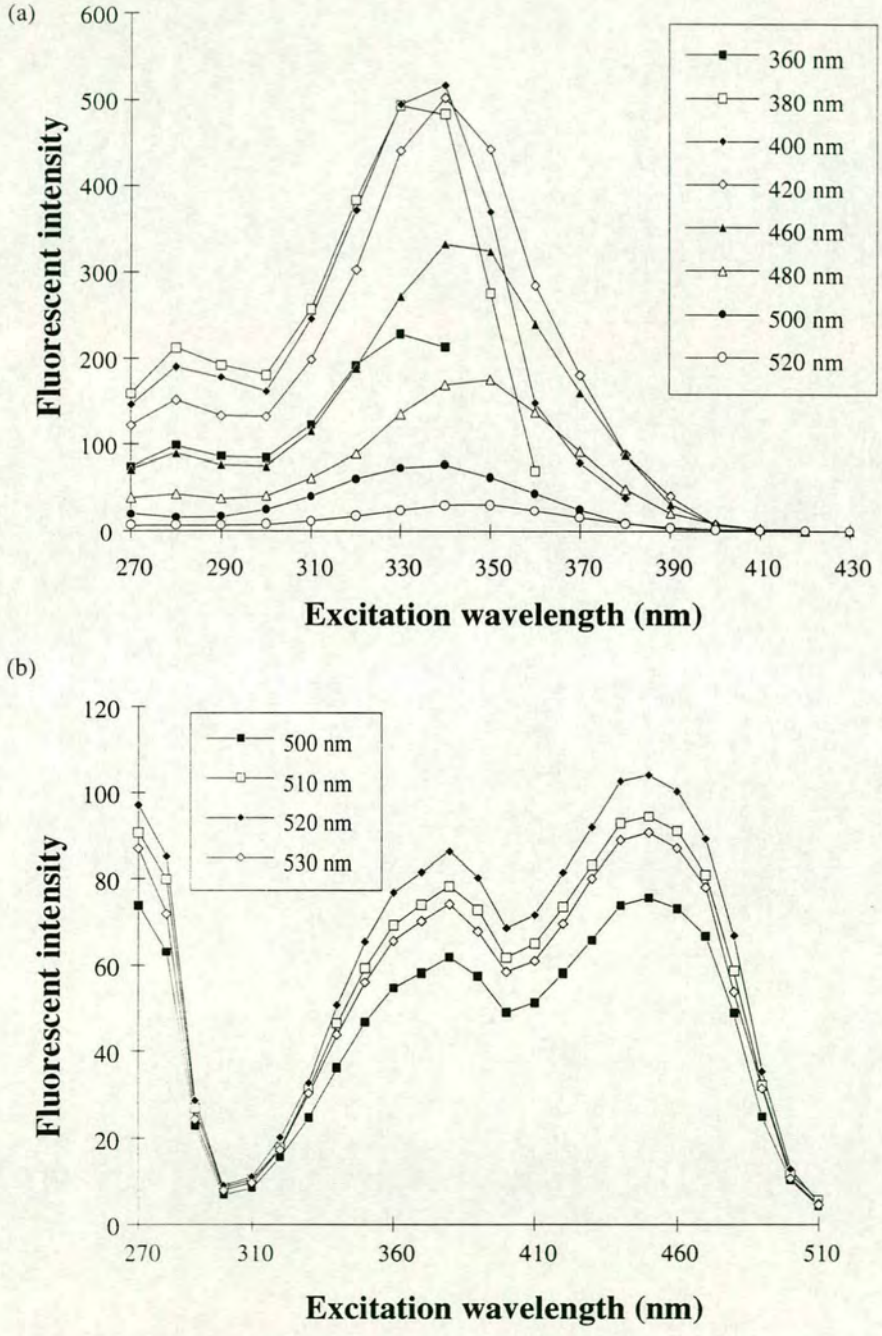


Figure 4.41 (a) Isoxanthopterin and (b) riboflavin EXEM spectrum. The concentration of the solution was 5.6 x10^{-7}M while the concentration of the riboflavin solution was 5.3x10^{-7}.

Although both of these excitation peaks are known to occur in coral solutions, the 280-285 nm excitation peak could also be due to tryptophan (see Figure 4.42, page 138), whilst the 340 nm peak is more likely be due to humic acid as Matthews *et al* in review have shown that humic acid quenches isoxanthopterin fluorescence. Thus, it is far from certain whether isoxanthopterin is a major contributor to coral fluorescence. The main excitation peak in riboflavin was at 450 nm, although smaller ones were also seen at 270 nm and 380 nm (see Figure 4.41b). The fact that the 450 nm excitation peak was not seen in any coral solutions suggested that riboflavin was not a major contributor to coral fluorescence.

Summary: Even though polyp tissue would appear to represent a very concentrated source of fluorophores, it was not possible to determine whether they were generated *in situ* or not. Although an excitation peak at around 340 nm has been identified in both coral solutions and polyp tissue, it is more likely to be due to humic acid than isoxanthopterin as Matthews *et al* (in review) have shown that the latter is quenched by the former. However, the fact that isoxanthopterin, and also riboflavin, were not positively identified in either polyp tissue or coral solutions, does not necessarily mean that they were not present (i.e. isoxanthopterin fluorescence could have been quenched by humic acids, while riboflavin may have only been present in low concentrations).

4.8.2 Marine organic matter: Although results in section 4.8.1 have suggested that polyp tissue fluorescence could be due to the *in situ* generation of fluorophores, it is generally thought that the majority of coral fluorophores come from the surrounding seawater (i.e. were ingested) (Boto and Isdale 1985, Susic *et al* 1991). Although this does not necessarily imply that these fluorophores were generated in the marine environment, a marine source of humic acids is certainly possible (see Appendix A1.1 for further details regarding the existence of a marine source of humic acids). Therefore, the aim of this section was to see whether: (1) a marine source could be identified, and (2) whether there was a difference in the fluorescent properties of seawater samples collected in the wet and dry seasons. For this reason, seawater samples were analysed.

Methods and materials: 0.5 litre seawater samples were collected from Aquarium Reef, Shark Bay, Porites Bay and Tin Smelter Bay adjacent to the living corals. A similar volume of water was also collected from a stream in Phuket Town. All samples were filtered and acidified before being shipped back to the UK. Excitation

began at 270 nm in an attempt to see any tryptophan fluorescence that may have been present as this has been reported in seawater by Coble *et al* (1990). EXEM spectra were produced for unconcentrated seawater and town water samples, and samples that had been concentrated from 200 mls to 20 mls. In addition to this, wet and dry season seawater samples were also compared. Dry season seawater samples were collected in March 1993 by Scoffin.

Results and discussion: It is acknowledged that the fluorescent characteristics of the seawater samples analysed in this chapter were unlikely to be truly representative for the following reasons:

(a) In order to obtain representative seawater samples, much larger volumes would be required. However, this would have created transport problems.

(b) Even though the samples were filtered and acidified, the three month shipping period would have affected the concentration and fluorescent properties of the organic molecules contained therein.

Bearing this in mind, results showed that all unconcentrated samples were very weakly fluorescent with the exception of water collected from a stream in the centre of Phuket Town. Although no distinctive humic acid features could be discerned, a weak excitation peak was seen at 275-285 nm (see Figure 4.42). Coble *et al* (1990) attributed a similar peak in their seawater samples to a protein-type fluorescence. The fluorescent signal of proteins has been commonly linked to the amino acids tryptophan (~285 nm) and tryosine (~275 nm) (Coble *et al* 1990), although a third amino acid, phenyl alanine, is also known to fluoresce (~255 nm, Lakowicz 1993). Although the 275-285 nm excitation peak seen in the Thai seawater samples could be caused by the presence of either tryptophan or tryosine, Lakowicz (1983) suggested that if both were present, fluorescence is more likely to come from tryptophan as it is excited at longer wavelengths (i.e. energy is transferred from tryosine to tryptophan as a consequence of NRET and/or RET). Although isoxanthopterin has an excitation peak in this region, the fact that it is rapidly degraded in seawater (Anita and Landymore 1975) effectively rules it out of the reckoning. Thus, the 275-285 nm excitation peak seen in the Thai seawater samples was most probably due to the presence of tryptophan. The fact that this peak was so poorly developed could be due to (1) a low tryptophan concentration (either naturally or as a consequence of

decay), and/or (2) the quenching of tryptophan fluorescence by humic acids (see Matthews *et al* in review for further details).

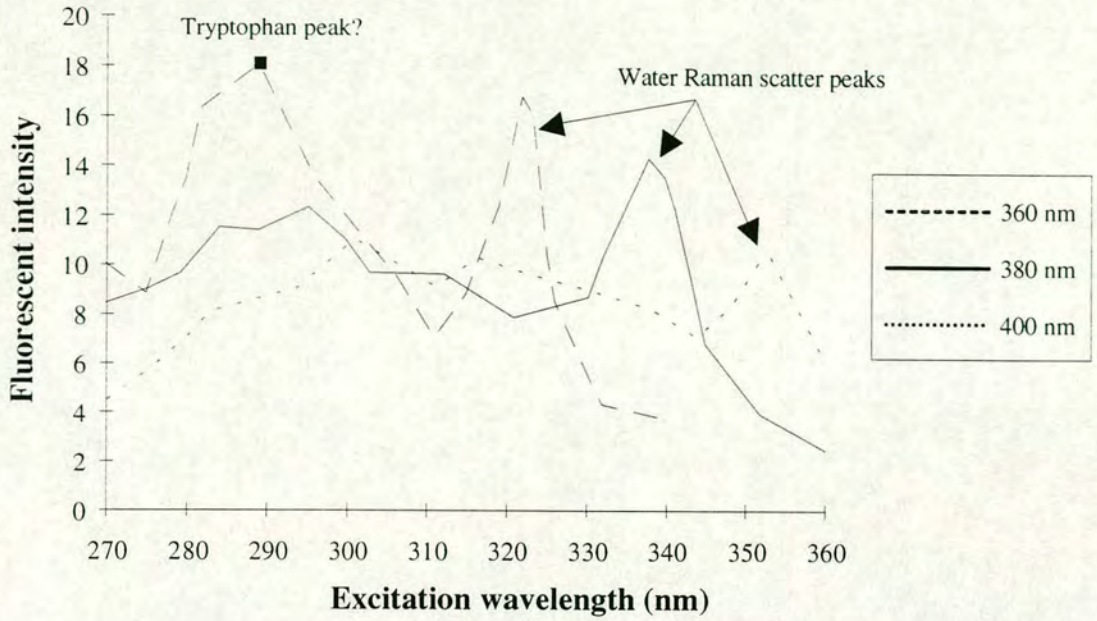


Figure 4.42 Seawater sample collected from Shark Bay (August 1992) showing 275-285 nm tryptophan excitation peak.

In general, when 200 mls of seawater was concentrated to 20 mls, the tryptophan peak disappeared and a fluorescence similar to that produced by humic acid became apparent (i.e. 330-345 nm excitation peak dominating the 390 nm excitation peak). The decrease in the size of the tryptophan peak probably reflects the increasing influence of humic acid quenching. When larger seawater samples were used (1200 mls concentrated to 20 mls), the 390 nm excitation peak became increasingly dominant (see Figure 4.43). Thus it appears that seawater contains the necessary fluorophores for coral fluorescence. The fact that tryptophan is known to be the dominant fluorophore in marine waters, and in particular surface waters, where it is thought to be associated with planktonic activity (De Souza Sierra *et al* 1994), therefore suggests that at least some of the fluorophores found in the Thai seawater samples could have been generated in the marine environment.

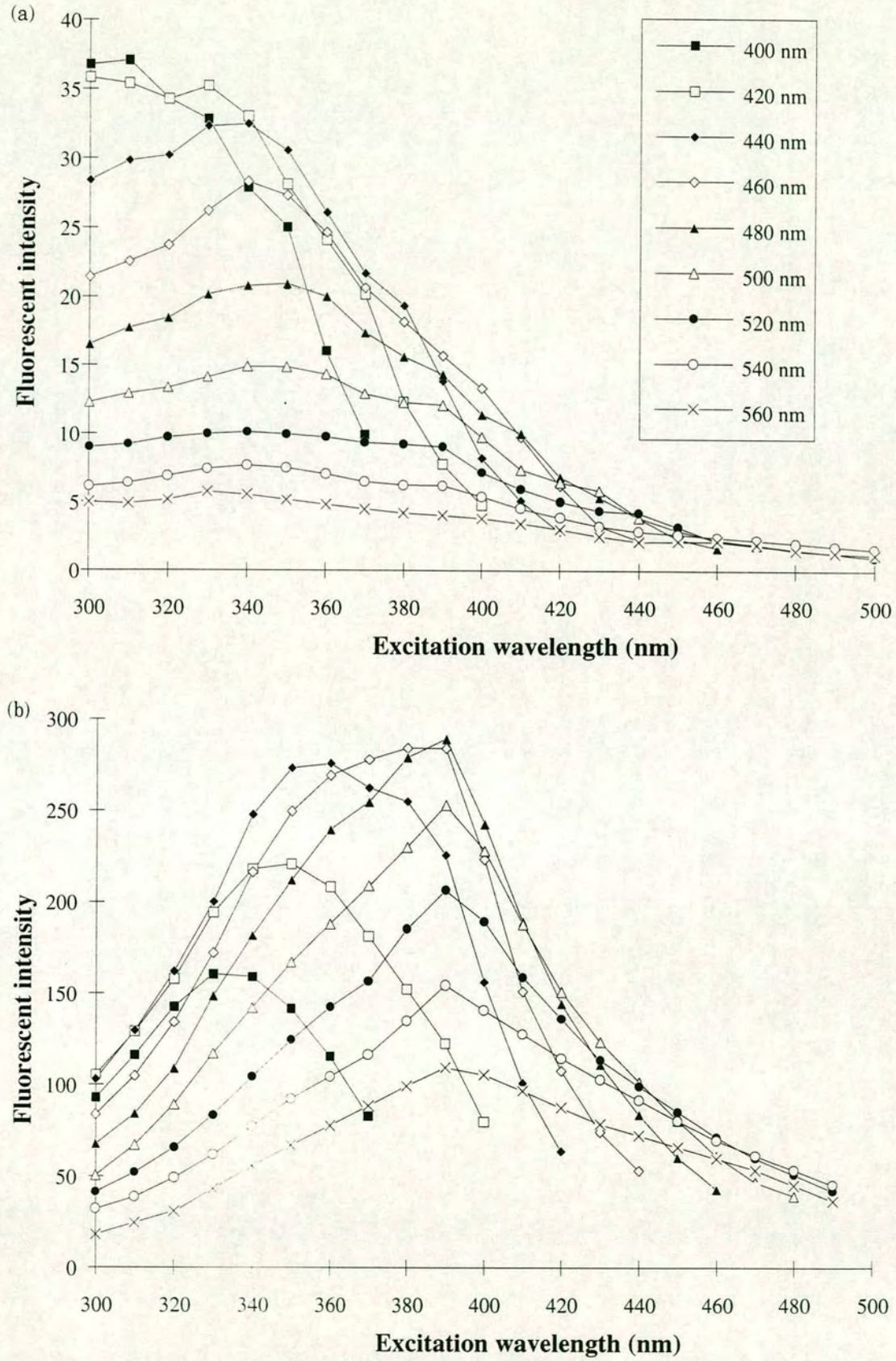


Figure 4.43 Phuket Town stream water collected in July 1993. (a) undiluted, (b) 1200 mls concentrated to 20 mls.

When wet and dry season seawater samples from the Thai area were concentrated from 200 mls to 20 mls, the concentration of fluorophores appeared to be higher in the wet season samples (see Figure 4.44).

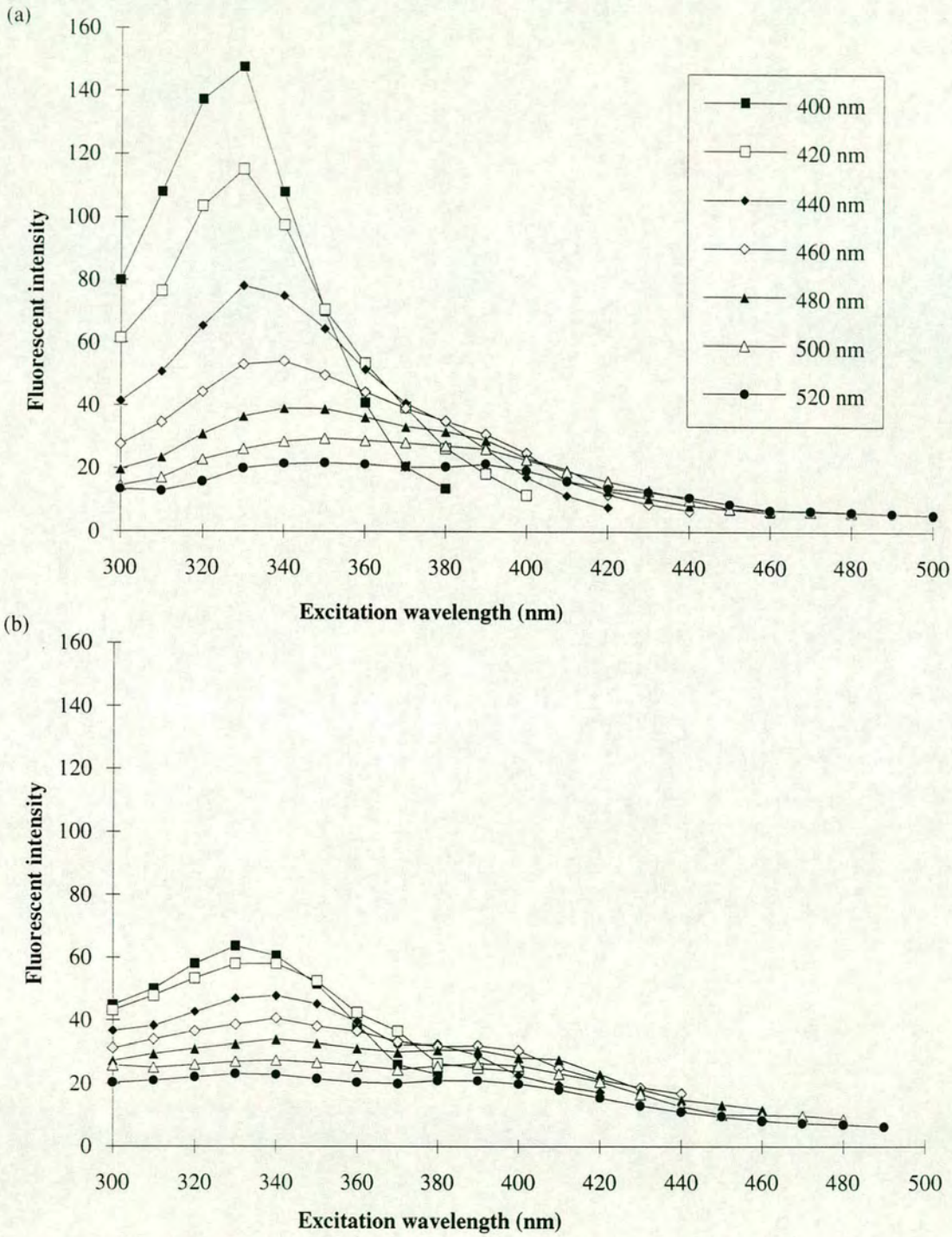


Figure 4.44 Wet versus dry season seawater EXEM spectra. (a) wet season sample and (b) dry season sample. Both samples were collected from Tin Smelter Bay. The wet season sample was collected by myself on 23.8.92 while the dry season sample was collected by Scoffin on 28.3.93.

This could be because more terrestrial fluorophores are washed into the marine environment during the wet season as suggested by Boto and Isdale (1985). Evidence to support this hypothesis was provided by Susic *et al* (1991) who found that river water humic acid concentration was approximately twice as high during the wet season. However, despite this apparent increase in the supply of terrestrial fluorophores into the marine environment during the wet season, dull bands are deposited at this time in the Thai study area. This apparent discrepancy is discussed further in Chapter 7.

Summary: Although the seawater around Ko Phuket contains the necessary fluorophores for coral fluorescence, the supply of which would appear to be greater during the wet season, it was not possible to determine whether the vast majority of the fluorophores responsible for coral fluorescence (i.e. humic acids) were generated in the marine environment or came from a terrestrial source. The fact that Susic *et al* (1991) found that the concentration of humic acid in river water was between 2 and 10 times greater than that in seawater, and that the concentration of humic acid in terrestrial soils was several orders of magnitude greater than in river water, would tend to suggest that a terrestrial source for coral fluorophores is very likely.

4.8.3 Terrestrial organic matter: Boto and Isdale (1985) suggested that terrestrial humic acids, washed into the marine environment during the wet season, were responsible for the bright bands seen in *Porites lutea*. For this reason, soil samples were taken to see if a terrestrial source of coral fluorophores could be identified.

Methods and materials: Locations and brief field descriptions are given in Figure 4.45. Soil and sediment samples were dried in an oven at 40°C before being shipped back to the UK for analysis. Once in the UK, they were sieved (180 μm mesh) in order to remove stones, leaves and twigs. Most methods involving the extraction of organics from soils have focused on humic acid extraction. As the majority of coral fluorophores appear to be humic acids, the method of Thurman *et al* (1988) for humic acid extraction was used. 0.5 g of soil/sediment was weighed into Teflon beakers and 5 mls of DH_2O was added so that no sample was lost during the evolution of gases during acid digestion. The samples were de-mineralised with 5 mls of concentrated (40%) HF. Although a number of fluorides are sufficiently volatile to be partly or completely lost when aqueous solutions are evaporated to dryness, many form quite stable fluoride complexes. 5 mls of concentrated HCl was then added, as the chlorine will displace the fluorine. The samples were then placed

on a sand bath and heated at approximately 160°C for 24 hours after which they were soaked in 25 mls of 0.5M NaOH for three days. The undissolved residue was filtered off before the liquid phase was evaporated down to 20 mls and adjusted to pH 9. As both concentrated HF and HCl are very strong acids, they may affect the fluorescent properties of the fluorophores. Thus, a similar set of experiments were carried out in which the samples were extracted with 0.5M NaOH directly.

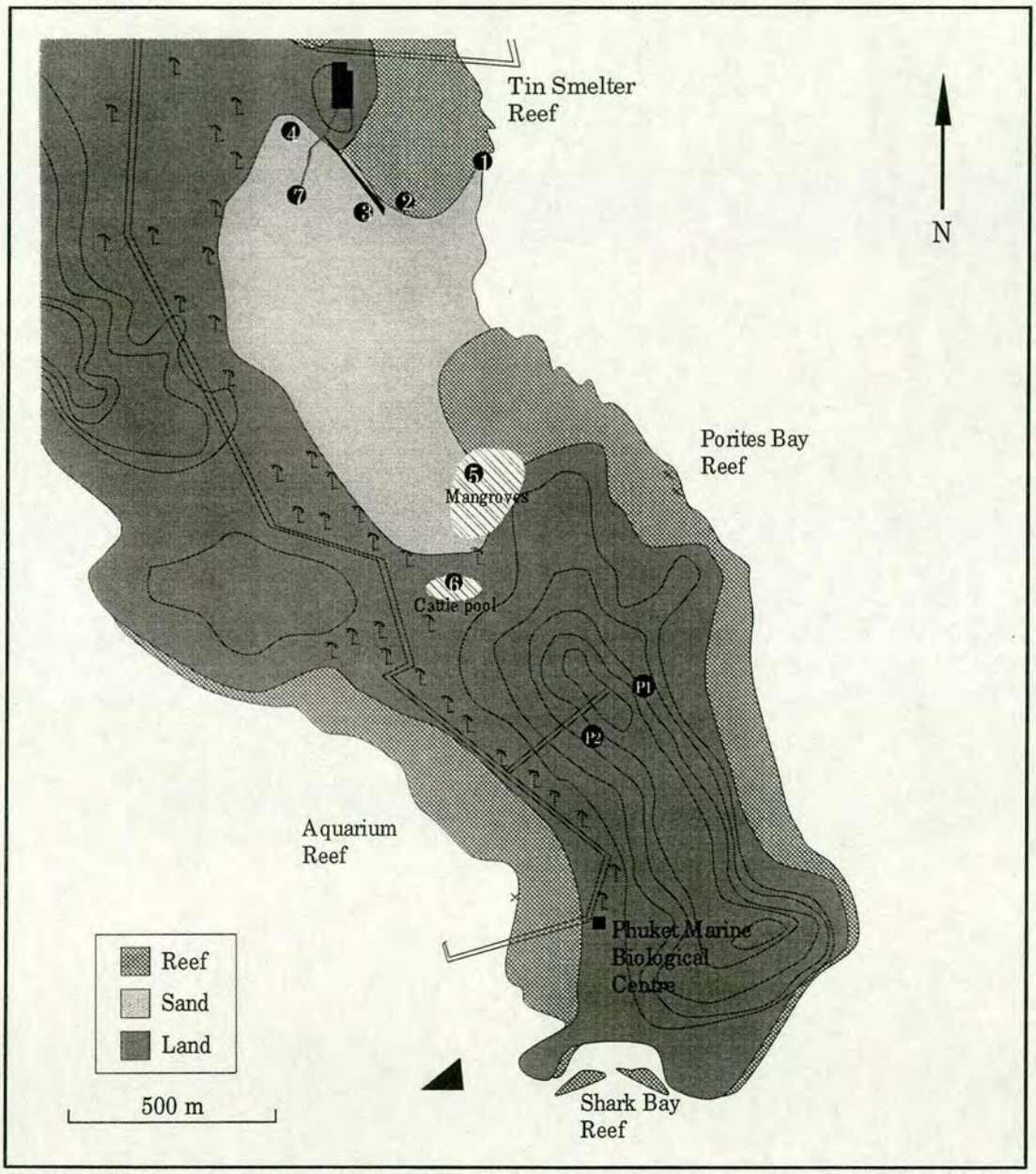
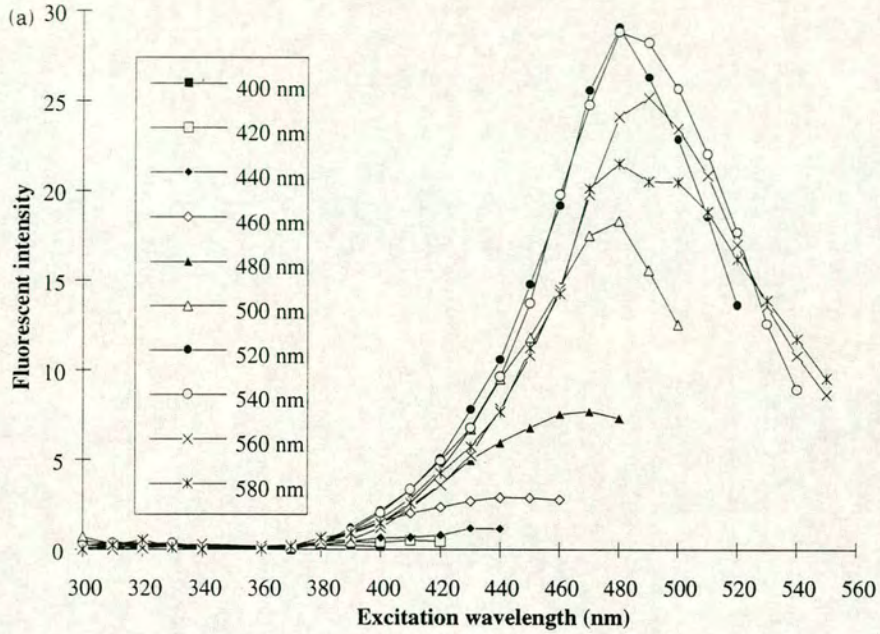


Figure 4.45 A map showing the location of soil and sediment samples. Key: 1-3: reef front sediment, fine-grained poorly consolidated carbonaceous sand containing clays; 4: beach sand; 5: mangrove sands, dark grey sand containing organic matter such as roots and leaves; 6: orange coloured mud from cattle pool; 7: stream by Tin Smelter, sands coloured black due to presence of illite and hematite; P1: Panwa Hotel profile 1; P2: Panwa Hotel profile 2.

Results and discussion:

(1) **Surface soil:** Results using a surface soil sample collected near the Panwa Hotel showed that while the HF/HCl extract was dominated by the 390 nm excitation peak, a pronounced 470-480 nm excitation peak dominated the NaOH extract (see Figure 4.46).



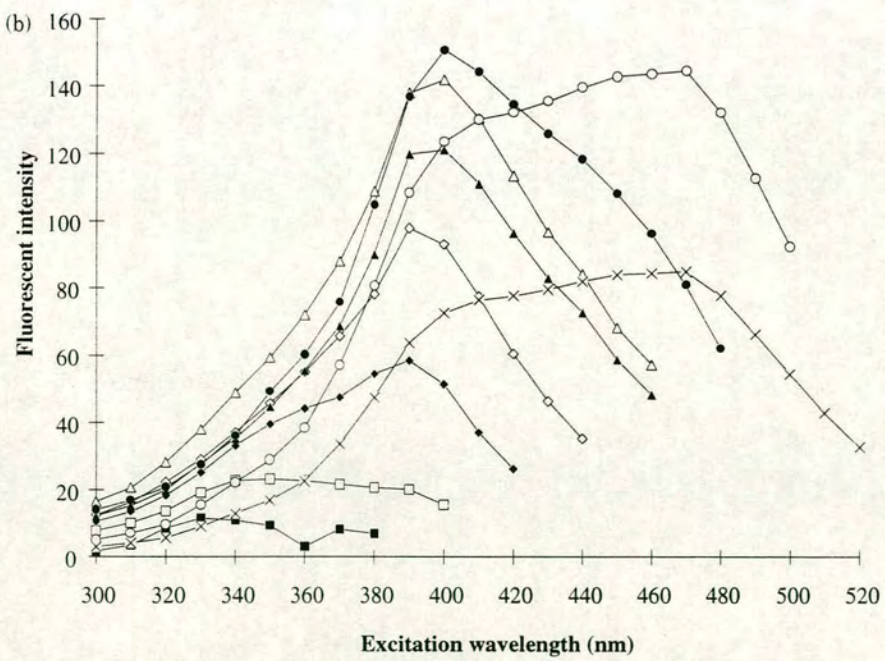


Figure 4.46 Surface soil extracts from Panwa profile (1). (a) extracted using 0.5M NaOH only, (b) extracted using HF and HCl followed by 0.5M NaOH.

Although the 470-480 nm excitation peak was not always as dominant as the one shown in Figure 4.46a, it was generally better developed in soils/sediments that were extracted with NaOH only. This could be due to one or more of the following:

(a) The concentration of fluorophores was higher in NaOH extracts. Significant differences in the colour of fluorescence were predicted when the surface soil extracts were excited at 330 nm, 360 nm, 390 nm and to a lesser extent 470 nm with the NaOH surface soil extract emitting fluorescence at longer wavelengths than the HF/HCl at each excitation wavelength (see Figure 4.47).

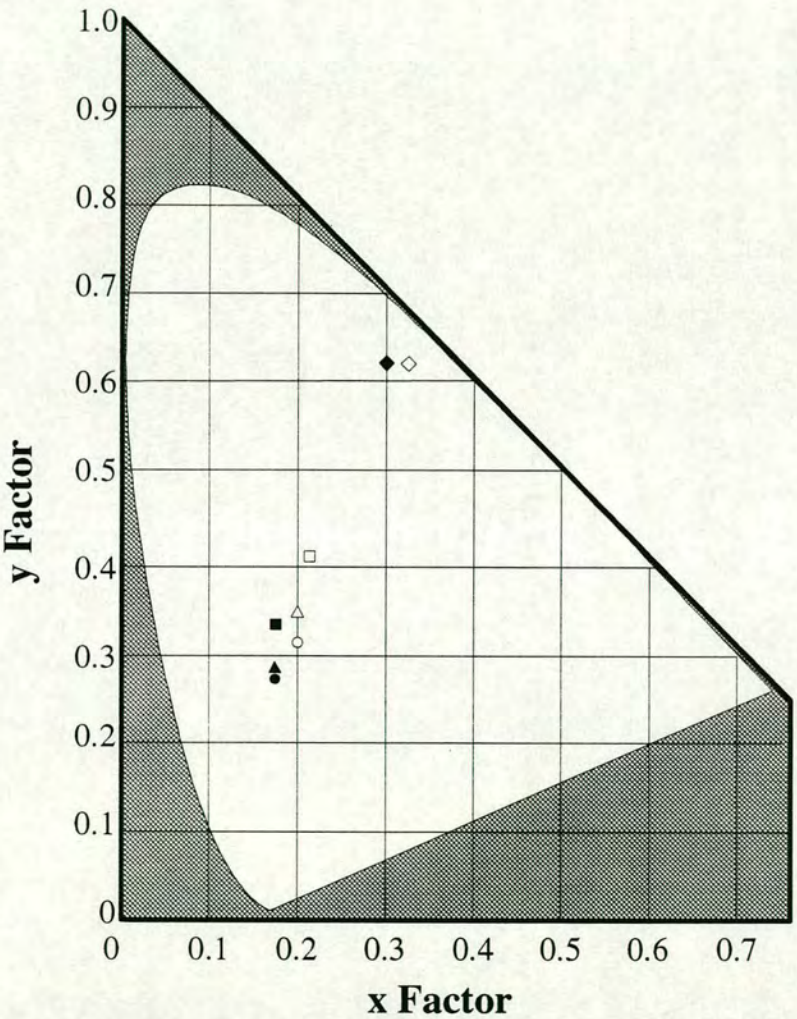


Figure 4.47 The affect that different extraction techniques have on the predicted colour of surface soil fluorescence. Key: black = HF/HCl surface soil extract, white = NaOH surface soil extract, circles = 330 nm excitation wavelength, triangles = 360 nm excitation wavelength, squares = 390 nm excitation wavelength, diamonds = 470 nm excitation wavelength. Refer to Figure 4.14 on page 88 to assign colours to these points.

Results in section 4.6.1 have shown that increasing the fluorophore concentration can, in some cases, result in both a shift towards longer wavelength emissions and a decrease in total fluorescent intensity (as a consequence of ET). Thus, the fact that NaOH surface soil extract emitted fluorescence at longer wavelengths and was less intense than the HF/HCl extract could mean that it contained a higher concentration of fluorophores. However, without knowing more about the precise nature of soil fluorophores and the effect of the different extraction techniques on their fluorescent properties, the differences seen in Figure 4.46 on page 143 can not be definitely attributed to concentration effects.

(b) HF destroys certain types of coral fluorophores.

(c) The high temperatures (160°C for 24 hours) used to remove the fluorine could destroy some of the fluorophores.

(d) NaOH dissolves silicates which are then free to complex with the fluorophores.

Humic acid complex formation: In order to test whether silicates were responsible for the 470-480 nm excitation peak, 0.025 g of sodium metasilicate was added to 4 mls of a soil extract (from the cattle pool) in which this peak was not seen. This resulted in the production of a pronounced 470-480 nm excitation peak whose increase did not appear to affect the intensity of the 330-345 nm excitation peak (see Figure 4.48).

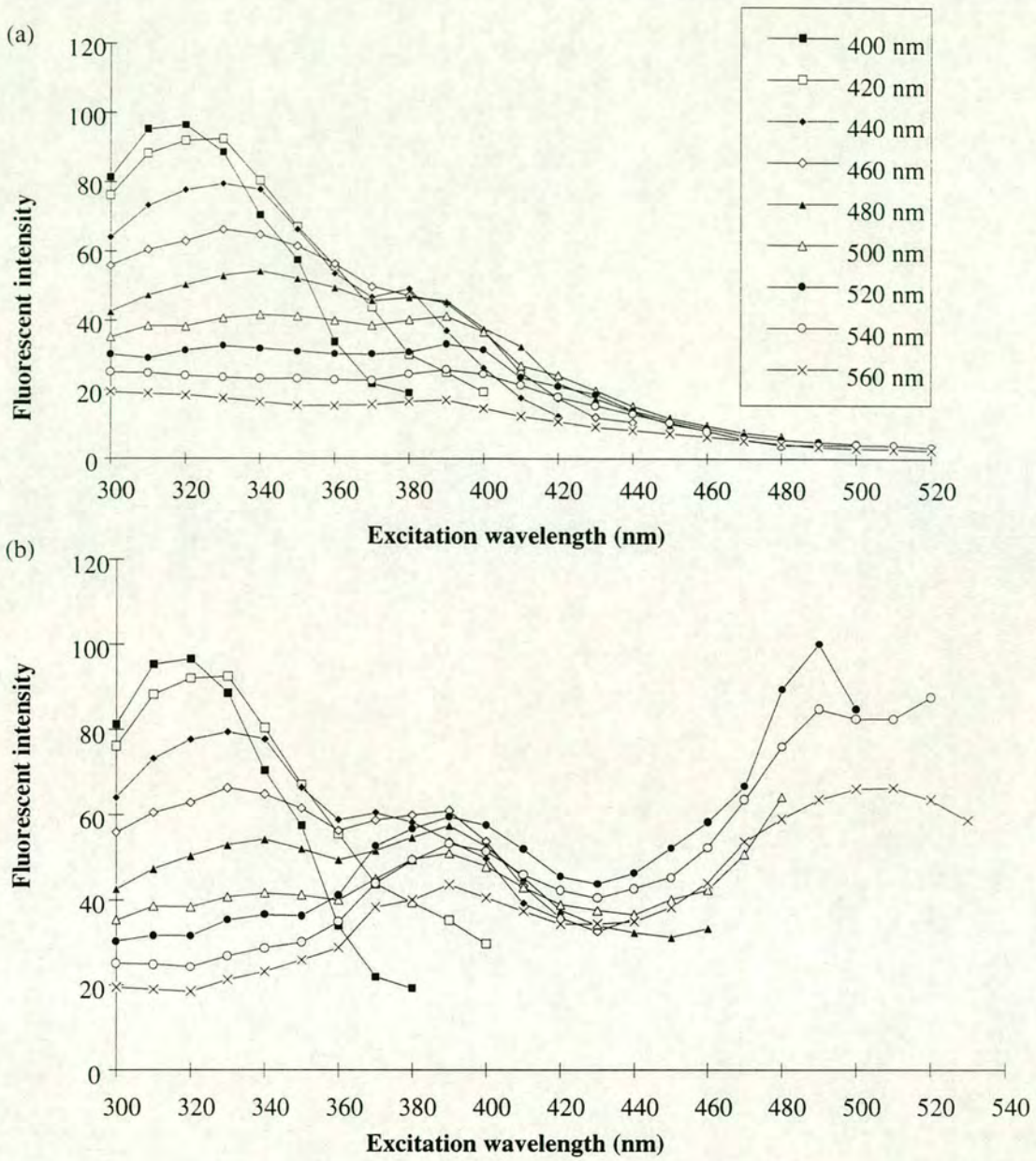


Figure 4.48 The effect of adding metasilicate to a soil extract collected from the Cattle Pool. (a) Soil extract standard (b) Soil extract spiked with sodium metasilicate.

These changes were not thought to be due to the presence of sodium metasilicate on its own as it was essentially non-fluorescent and did not absorb between 300 nm and 500 nm. Sodium ions were also not thought to be involved in this process as no significant difference could be detected when 2.5 g of NaCl was added to 20 mls of a 4M coral solution (see Figure 4.49). These results therefore suggested that a complex had formed between the metasilicate and possibly, although not necessarily, humic acid. If the 470-480 nm excitation peak seen in soil extracts was due to silicates, then silica (i.e. sand) and clay were the most likely sources in these coastal

settings. However, as results in Chapter 3, section 3.3, page 55, have suggested that the presence of certain clays can inhibit coral fluorescence, silica was the more likely source. The 470 nm excitation peak, which occurs in Aldrich and Fluka humic acid EXEM spectra, could therefore be due to the formation of a complex between humic acid and the humic acid impurities which are known to be present. The fact that this excitation peak was still seen in Fluka humic acid extracts (i.e. after having passed through XAD-2 resin) could be because the resin failed to remove all the impurities.

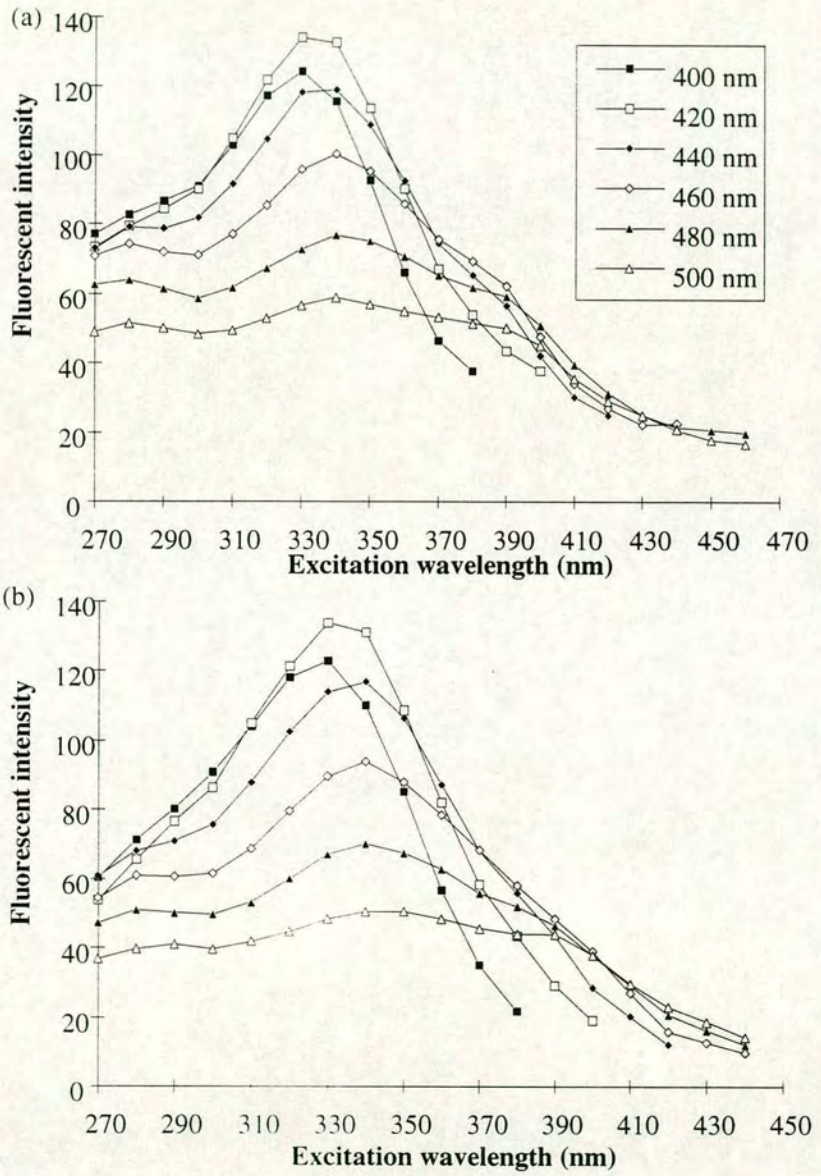


Figure 4.49 The effect of sodium ions on coral fluorescence. (a) No sodium (b) 2.5g of NaCl in a 20 mls 4M coral solution.

One possible complex that was common to both the NaOH and HF/HCl extracts was identified in sediment collected from the stream next to the tin smelter. The EXEM

spectrum looked very similar to typical 1M coral solution EXEM spectra in all respects except for the appearance of an excitation peak at 530 nm (see Figure 4.50).

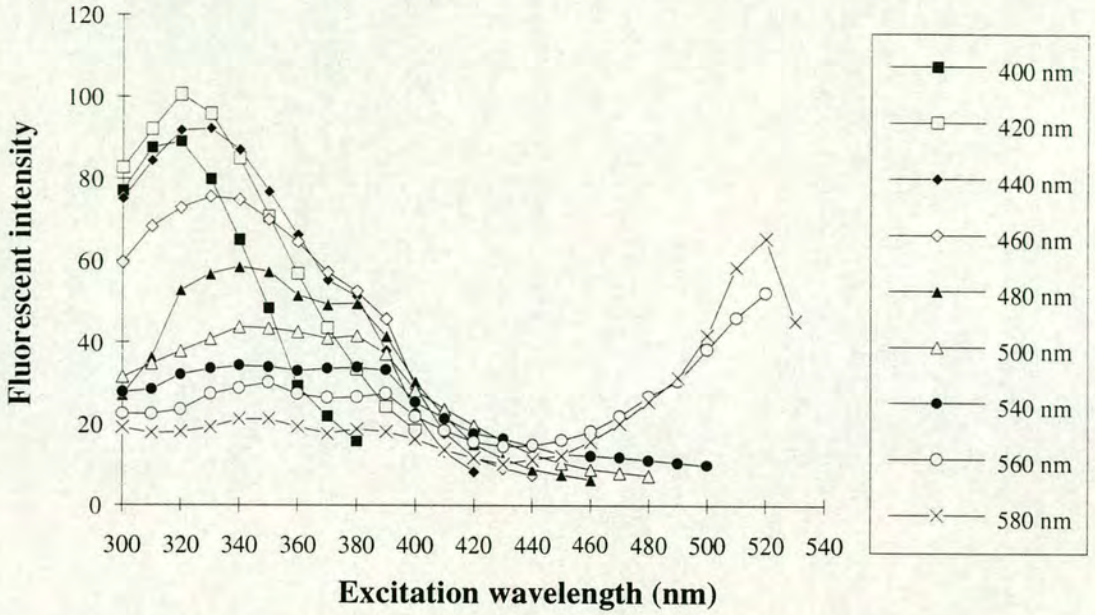


Figure 4.50 Soil sample collected from the Tin Smelter discharge stream.

This excitation peak was not detected in any other soil sample, coral solution or solid coral sample (see Chapter 6, Figure 6.2, page 171 for a solid coral EXEM spectrum) and was thought to be associated with the high levels of illite and hematite (confirmed by XRD) which are discharged from the tin smelter and give the stream sediment its distinctive black colour.

To summarise, although surface soil extracts more closely resemble concentrated Fluka humic acid solutions than coral solutions, there are a number of differences, namely the presence of the 470-480 nm and 530 nm excitation peaks and the absence of a 275-285 nm excitation peak. Although the 470-480 nm and 530 nm excitation peaks could be due to complex formation between humic acid and silicate, the lack of such excitation peaks in coral solutions or in the solid state could simply be because coral skeleton, which is free from the visible signs of borings, contains virtually no silicates. The lack of a 275-285 nm peak does not, however, provide irrevocable proof that the tryptophan excitation peak seen in seawater samples is marine in origin. This is because humic acid has been shown to quench tryptophan fluorescence (Matthews *et al* in review). As the concentration of humic acids is very high in these surface soil samples, tryptophan fluorescence is unlikely to be seen.

(2) **Reef front and mangrove samples**: 0.5 g sediment samples collected from the reef front were extracted in the same way as the surface soil samples. The fact that their EXEM spectra closely resembled typical 1M coral solution EXEM spectra (see Figure 4.51) suggested that the concentration of fluorophores in these reef front sediment samples was significantly lower than in inland surface soils.

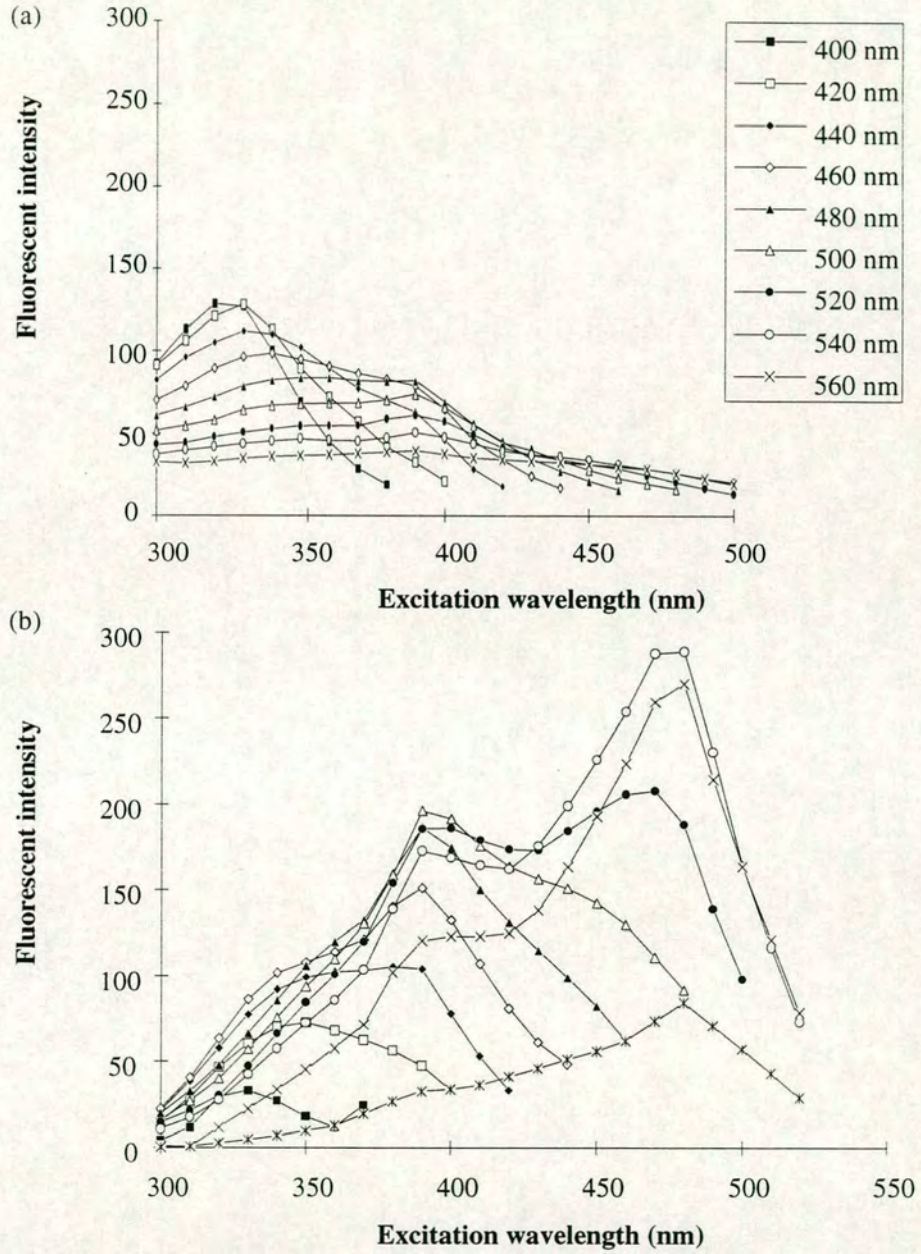


Figure 4.51 Reef front sediment (from site TS-5 collected in 1993) extracted with NaOH and concentrated to (a) 20 mls and (b) 4 mls.

This was confirmed when the reef front sediment extracts were concentrated to 4 mls (see Figure 4.51b), as a strong 470-480 nm excitation peak appeared. It was

originally thought that the mangrove swamps at the head of Phangnga Bay were the principal source of terrestrial humic acids. However, when a 0.5 g sample taken from the mangrove sands in Porites Bay was extracted, the EXEM spectrum was dominated by the 330-345 nm excitation peak and no 470-480 nm excitation peak was found. Concentrating to 4 mls merely resulted in an increase in total fluorescent intensity but no significant change in the shape of the EXEM spectrum. When a 2.5 g mangrove sample was extracted, the 470-480 nm peak appeared. Thus, although the mangrove swamps could be a potential source of fluorophores, surface soil represented a more concentrated source of the same or very similar fluorophores. As was the case with surface soil extracts, the 275-285 nm tryptophan peak was not seen even when the extracts were diluted. However, as mentioned before, this could be due to humic acid quenching.

Summary: Although all soil and sediment samples analysed contained the fluorophores necessary for coral fluorescence, the concentration varied between sites, decreasing from inshore surface soils to reef front sediments. In addition to the fluorophores commonly found in coral solutions, soil samples contained two extra fluorophore groups, one at 470-480 nm and a second (although much less common) at 530 nm, both of which were attributed to the formation of a fluorescent complex between humic acid and silicate. However, their absence in coral solutions and solid coral could be because: (1) the concentration of these fluorophores was not sufficiently high for their fluorescence to be detected, and/or (2) unaltered parts of the coral skeleton contained virtually no silicates for complex formation. Although the absence of a tryptophan peak in surface soil extracts could imply a marine origin for 275-285 nm excitation peak seen in some coral solutions, quenching by humic acid could be the reason why it was not seen.

4.8.4 Implications for coral fluorescence: In the Thai study area, seawater, soil and sediment EXEM spectra looked very similar to coral solution and solid coral EXEM spectra once concentration differences were taken into account. Because of this, it was not possible to determine whether one or many fluorophore sources existed. The fact that the inland surface soil extracts had the highest concentration of coral-like fluorophores and that concentrations decreased with increasing distance from these sites, suggested that inland surface soil was the major source of coral fluorophores in this area. This would therefore imply that the fluorophores found in seawater samples and the sediments near the reef were transported there from an inland source. However, the presence of the 275-285 nm tryptophan excitation peak seen in

some coral solutions and seawater samples and its absence from terrestrial samples could be used as proof that marine fluorophores contribute towards coral fluorescence. However, without ^{13}C data (marine plankton is, on average, depleted in ^{13}C compared with terrestrial humic acids, Nissenbaum and Kaplan 1972), it was not possible to determine whether the fluorophores found in the coral skeleton were derived from a terrestrial or marine source, if indeed they were generated outside the coral polyp.

4.9 CONCLUSIONS:

(A) By using EXEM spectra to study coral fluorescence, a more detailed fluorescent fingerprint can be produced than when emission spectra are used. At present this technique enables two, but possibly even three, fluorophore groups to be identified in coral solutions (330-345 nm, 390 nm, and a possible excitation peak at 275-285 nm).

(B) The fluorescent colour of industrial humic acid solutions appears to be related to the concentration of fluorophores in the solution. As the fluorophore concentration increases, colour shifts to longer wavelength emissions (i.e. blue to yellow). This change in colour has been attributed to ET processes and the presence of inorganic impurities which absorbed more strongly at shorter wavelengths therefore preferentially absorbing blue light and thus allowing more green light to be emitted. However, when bright and dull band coral solutions were compared, no significant differences in the EXEM spectra could be detected. This was attributed to: (1) the poor response of the PMT to emissions over 580 nm which meant that both green (y) and red (x) component light would be underestimated, and (2) because the concentration of fluorophores in coral solutions was low. The only real difference detected between bright and dull band solutions was one of fluorescent intensity with bright band fluorescent intensity being greater. Results using Fluka humic acid solutions showed that below the ET threshold (i.e. less than between 36-14 ppm humic acid), increases in concentration resulted in a more or less linear increase in fluorescent intensity at all wavelengths. As the concentration of fluorophores in coral solutions appeared to be below the ET threshold, the fact that bright band intensity was equal to or greater than dull band intensity suggested that the concentration of fluorophores in bright bands was equal to or greater than the concentration in dull bands.

(C) The presence of the metal ions Fe^{3+} and Mn^{2+} have been shown to reduce (quench) coral fluorescence by preferentially absorbing short wavelength emissions. Whilst the levels of manganese found in corals of a related family were not considered high enough to affect coral fluorescence (0-15 ppm), the levels of iron (0-210 ppm) were. However, no evidence was found to suggest that the concentration of iron varied seasonally (Allison 1994). This therefore suggests that whilst the presence of iron might affect fluorescent intensity, it is unlikely to be the main cause of fluorescent banding in *Porites lutea* from Ko Phuket.

(D) Although XAD-2 resin has often be used to extract humic acids from soil and seawater samples, results in this chapter have demonstrated that this resin should not be used when studying coral fluorescence for the following reasons: (1) the resin is very difficult to clean effectively which is of particular importance considering the impurities it contains have excitation peaks in the same places as coral fluorophores, (2) the resin is either quickly saturated or not very efficient as the residue phase is often as fluorescent as the extract, and (3) the resin selectively absorbs humic acids and hydrophobic molecules and may therefore exclude some of the fluorophores that are involved in coral fluorescence.

(E) The fluorescence seen in corals of the species *Porites lutea* from Ko Phuket appears to be due to the incorporation into the skeleton of humic acid-like fluorophores. However, it is uncertain from where these fluorophores are derived, as seawater, surface soil, reef sediment and polyp tissue all have the necessary characteristics of coral fluorescence. Even though the concentration of fluorophores in soil and sediment samples appeared to decrease with increasing distance from the mainland, this does not necessarily imply that terrestrial surface soil is the main source of coral fluorescence as these fluorophores could just as well have been produced in the marine environment or in the polyp tissue. Thus, a present, it was not possible to identify exactly where the coral fluorophores are generated. The presence of the 275-285 nm excitation peak found in seawater and some coral solutions could suggest that at least some of the coral fluorophores were marine in origin as this peak was not found in terrestrial soil samples. However, this peak could also be due to isoxanthopterin, produced in the polyp tissue by the polyp itself or by its zooxanthellae.

CHAPTER 5. THE FLUORESCENT CHARACTERISTICS OF CORAL SKELETON WHEN VIEWED USING A FLUORESCENT MICROSCOPE

CHAPTER 5. THE FLUORESCENT CHARACTERISTICS OF CORAL SKELETON WHEN VIEWED USING A FLUORESCENT MICROSCOPE

5.1 INTRODUCTION:

Fluorescence microscopy is widely used to characterise the organic matter in recent and ancient sediments, petroleum source and reservoir rocks, oil shales and coal. Its use stems from the fact that when an electron is excited by u/v or visible light, the fluorescence that is emitted when the electron decays back down to the ground state depends to a large extent on what sort of transition the electron undergoes and from where this electron comes from (i.e. what atom or atoms it is associated with and whether it is associated with a bonded or non-bonded orbital). For a full review of bonding and non-bonding orbitals and the types of transition that can occur between them, see Coyle (1989). Orbital configuration mixing, energy transfer processes (discussed in Chapter 4, section 4.6.1, page 97) and intermolecular interactions can all occur in a complex mixture of organic compounds, and often result in a highly complicated pattern of emissions. Although the study of these patterns has facilitated some degree of characterisation, more work needs to be done before spectral ranges for specific chemical structures in highly complex organic matter can be assigned. However, as far as coral fluorescence is concerned, work in this chapter has concentrated on examining the distribution of fluorophores in the coral skeleton rather than on the structural and chemical composition of the organic molecules that give rise to the fluorescence.

5.1.1 Background: Fluorescent microscopy was first used to study coral fluorescence by Quale (1991) using *Porites lutea* collected from Thailand by Tudhope and Scoffin. Results showed that fluorescent intensity was heterogeneous within a band which, Quale suggested, reflected the complex interaction between the incorporation of organic matter into the skeleton and skeletal growth. In addition, her results demonstrated that although bright band fluorescent intensity was up to 100% more intense than dull band fluorescent intensity, the emission spectra were similar, maximising at around 450 nm when excited at 480 nm. She therefore suggested that despite the apparent difference (to the eye) between bright and dull bands, the same materials were responsible for fluorescence in each band. These findings are therefore in keeping with results in Chapter 4, section 4.4.2, page 82, in

which no difference was detected in the shape of bright and dull band excitation/emission (EXEM) spectra. The fact that Quale notes that bright fluorescent bands are up to 100% more intense than dull bands would suggest that the concentration of fluorophores in the solid state is below the energy transfer (ET) threshold. However, estimates of the concentration of humic acids in the coral skeleton made in Chapter 4 (see section 4.6.1, page 109), by Matthews *et al* (in prep) and Susic *et al* (1991) all indicate levels above the theoretical ET threshold. This may therefore suggest that it is the manner in which the fluorophores are bound in the skeleton that is crucial in determining fluorescent banding. Thus, the aim of this chapter is to characterise the spatial distribution of fluorophores within the skeleton at the microscopic level.

5.2 METHODS AND MATERIALS:

Fluorescence was viewed using a Zeiss fluorescence microscope which has a realistic maximum magnification of approximately x600. However, even at this magnification it was not possible to characterise the inter-crystalline spatial distribution of fluorophores. This was because the aragonitic fibres in *Porites lutea* are between 0.25 μm and 0.5 μm in diameter whereas the field of view was ~55 μm. Initial work used cleaned coral skeleton which had been polished using 200 and then 600 grade carborundum paper. However, because of the porous nature of the skeleton, the vast majority of the sample was out of focus at any one time. As a consequence of this, it was not possible to determine whether fluorescence originated from specific areas or, as it appeared, from all parts of the skeleton fairly equally. To avoid this problem, pore spaces were filled with a non-fluorescent medium. Good results were obtained with black epoxy resin (supplied by B and K resins).

Before making thin sections, the position of the fluorescent bands were identified under a u/v lamp and recorded on tracing paper. As glass is relatively impervious to u/v light below 360 nm, a cover slip was not added to the thin section. To prevent minute particles of dust accumulating on the slide while the epoxy resin was setting, the slide was placed in a dessicator. It was important to do this as dust particles were thought to be responsible for numerous small, but very bright blue fluorescent patches (an example of which is provided later). Samples were excited with 360 nm u/v light and photographs were taken using Kodachrome T64 film. Filters were used to prevent u/v light from the lamp reflecting off the coral surface and being

seen/recorded in photographs. The colour on the film depends to a large extent on the film used. With fluorescence, it is very difficult to get close to the original colour observed (Bishop *pers. comm.*). However, Kodachrome T64 was found to give reasonable colour rendition.

5.3 RESULTS AND DISCUSSION:

5.3.1 The nature of coral fluorescence seen using a fluorescence microscope: When viewed using a fluorescence microscope, all parts of the coral skeleton emitted fluorescence. Within both band types, fluorescence was not considered to be homogeneous and could be broadly divided into two main components:

(a) **Irregularly shaped yellow/orange fluorescent patches**: The majority of these patches were approximately 15 μm in diameter although they ranged in size from 8 μm to 35 μm in diameter (see Figure 5.1). These patches were randomly distributed throughout the skeleton.

(b) **Background region**: This was generally blue in colour (various shades) with a distinctly mottled appearance.

Work in Chapter 2 (see Figure 2.5, page 33), has shown that holes and grooves artificially cut into the coral skeleton appear to increase fluorescent intensity as a consequence of increased surface area. However, when cracks were examined using a fluorescence microscope, they bore no relationship to the yellow/orange fluorescent patches (see Figure 5.1a and b). Thus, at the microscopic level, the yellow/orange fluorescent patches must be due to something else. When the yellow/orange fluorescent patches were examined in transmitted light, the fluorescence appeared to originate from relatively small (approximately 5-6 μm in diameter), dark possibly organic areas. Assuming this to be the case, it would appear that the fluorescence emitted from these organic inclusions illuminates a relatively large area. As a consequence of this, some of the shift to shorter wavelengths in the areas around the yellow/orange patches could be attributed to a decrease in the intensity of yellow/orange fluorescence away from the dark area and an increase in the contribution made by the background fluorescence. The background region of the skeleton would also appear to contain organic material although the patches were too small to see individually (i.e. sub-micron). As observations suggest that the least

fluorescent areas of the skeleton are the cleanest/whitest areas in transmitted light, it would appear that the greater the apparent darkening of the coral skeleton in transmitted light (presumably brought about by a greater amount of organic material), the brighter and lighter the fluorescence.

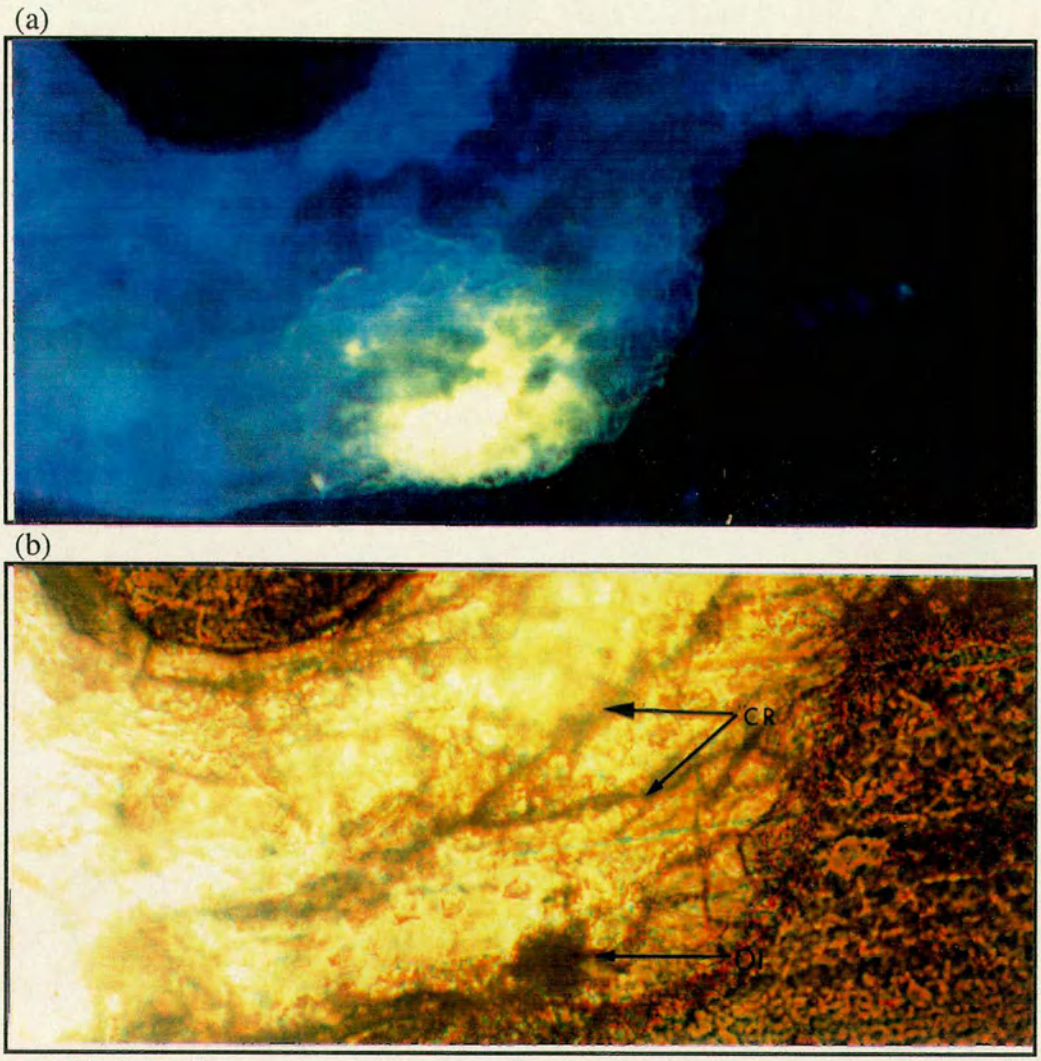


Figure 5.1 Coral fluorescence seen under a fluorescence microscope: see overleaf for figure caption.

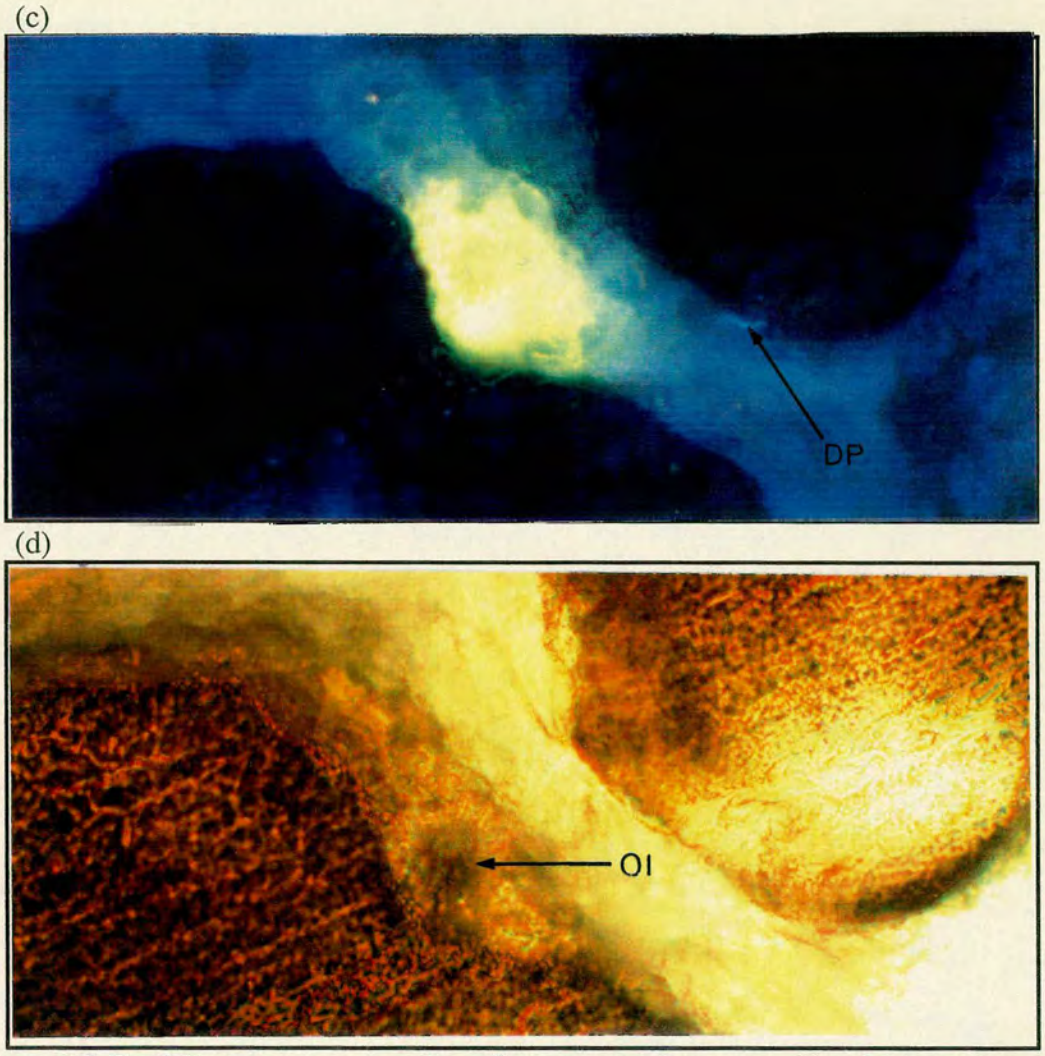


Figure 5.1 Coral fluorescence seen under a fluorescence microscope: (a) and (c) are photos of parts of the coral skeleton taken under u/v light while (b) and (d) respectively are the corresponding transmitted light photographs. CR = cracks, OI organic? inclusion, DP = dust particle. The colours seen in this, and all subsequent photographs in this section were not identical to those seen under the fluorescence microscope. For example, the pore spaces should have been black as they were infilled with black epoxy resin. This therefore means that (1) the yellow/orange patches are bluer than they should be and (2) that the background fluorescence is not as intense as it appears. These effects were attributed to (1) the developing process and (2) incomplete removal of u/v light. As these photographs were taken at a magnification of x600 using 35 mm film, the width of view is approximately 58 μm (i.e. 35 mm divided by 600). The yellow/orange patch in (a) is approximately 12 μm in diameter and appears to originate from a dark inclusion with a diameter of approximately 6 μm (see photograph b). The yellow/orange patch in (c) is approximately 18 μm in diameter and appears to originate from a dark inclusion approximately 6 μm in diameter (see photograph d).

Emission spectra were obtained for a number of these yellow/orange fluorescent patches which, when compared to the background blue fluorescence, emitted a significantly higher proportion of longer wavelength emissions (see Figure 5.2).

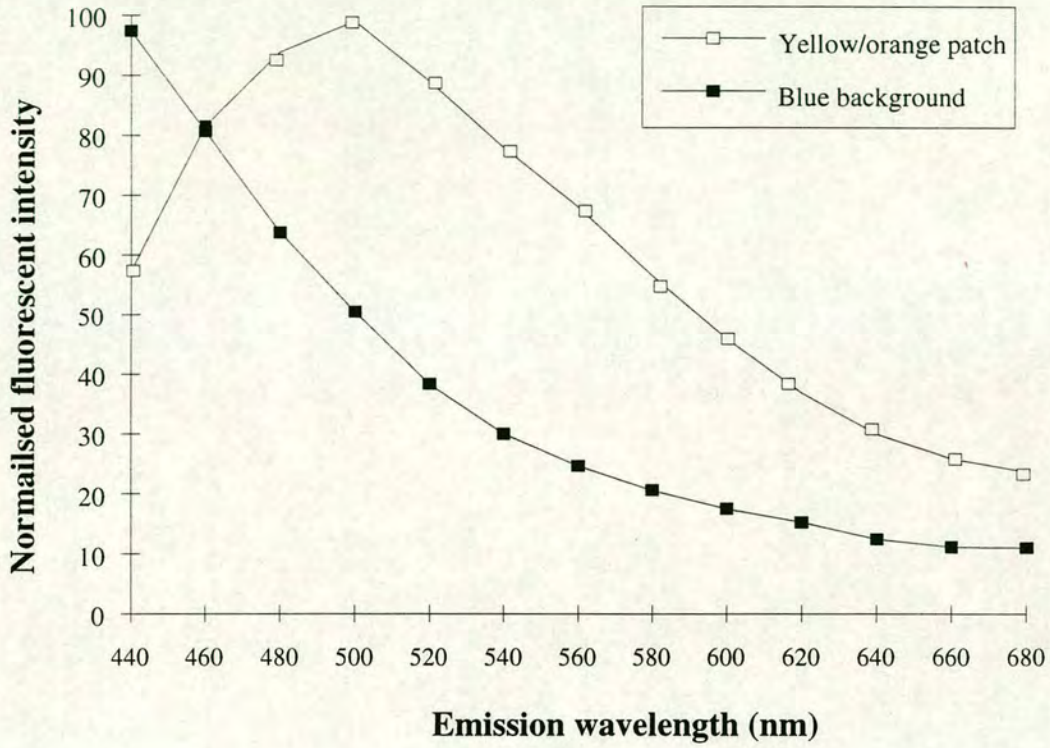


Figure 5.2 Emission spectra of (a) a yellow/orange fluorescent patch and (b) background fluorescence. It should be noted that it was not possible to determine the relative intensity of background fluorescence and yellow/orange fluorescence because the fluorescence microscope only measured relative intensities. Therefore, it was only possible to compare the shape of the emission spectra.

In addition, the shape of the emission spectra from the yellow/orange fluorescent patchs are very similar to both the bright and dull band solid state emission spectra presented by Boto and Isdale (1985) (see Chapter 1, Figure 1.3, page 6) which they attributed to humic acids. Thus, the large yellow/orange fluorescent patches could be due to humic acid or humic acid-like fluorophores.

Observations also suggest that the yellow/orange spots are brighter than the background region of the skeleton. Bearing in mind that fluorophore concentrations above the ET threshold are also associated with a reduction in fluorescent intensity (see Chapter 4, Figure 4.23, page 103), perhaps this suggests that the concentration of fluorophores in the background region of the coral is very low. Figure 5.3 describes a situation in which it is possible for fluorophore concentration to be both above the

ET threshold and more intense than certain fluorophore concentrations below the ET threshold.

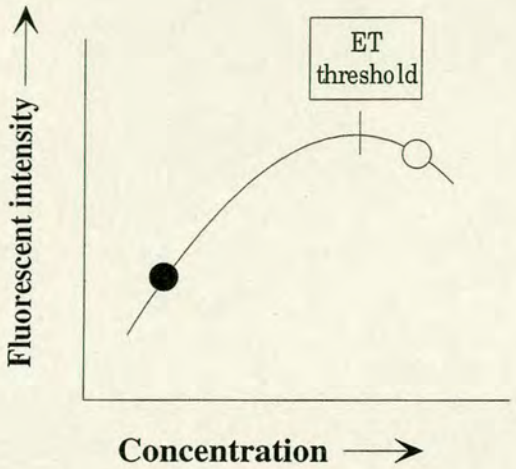


Figure 5.3 Schematic diagram showing how fluorescent intensity of coral fluorophores changes with increasing concentration. The fluorescent intensity of the white circle is above that associated with the black circle even though its fluorophore concentration is higher. In Chapter 4, section 4.6.1, page 102, it was noted that below the ET threshold, increases in the concentration of fluorophores resulted in an increase in fluorescent intensity. However, when the concentration of fluorophores increased above the ET threshold, a decrease in fluorescent intensity was recorded (see Chapter 4, Figure 4.23, page 103). This change in fluorescent intensity with increasing concentration is displayed schematically in this figure.

Finally, the large, possibly organic, inclusions which give rise to the yellow/orange fluorescence (see Figure 5.1b and d on pages 157 and 158), are too big to be found within a single aragonitic fibre, which in *Porites lutea*, are between 0.2 μm and 0.5 μ m in diameter (see Figure 5.4).

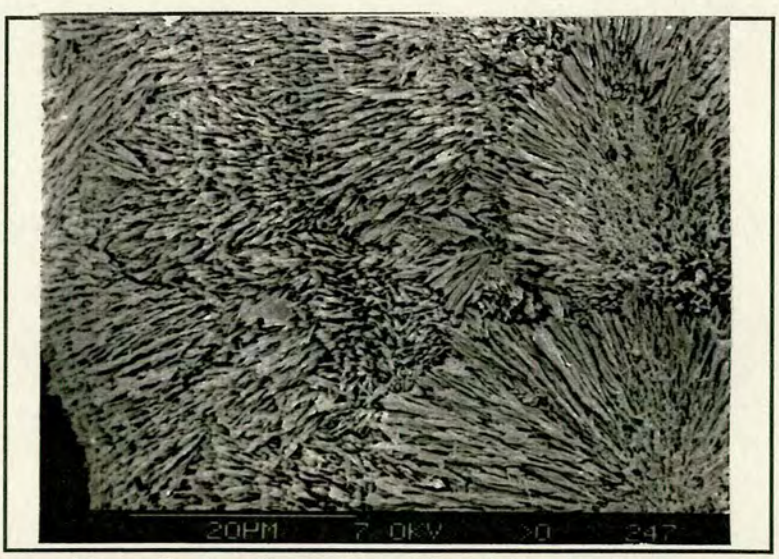


Figure 5.4 Aragonitic needles in a *Porites lutea* collected from Porites Bay seen using a scanning electron microscope. The sample was briefly etched in weak HCl to enhance detail.

Thus, these organic inclusion are more likely to be inter-crystalline than intra-crystalline. This raises the question of fluorophore oxidation. Presumably the aragonite needles can grow around the organic inclusions thus effectively isolating them. This would appear to be the most logical conclusion considering these yellow/orange patches are present after cleaning with NaOCl. Although the location of the sub-micron sized inclusions was not determined, they could conceivably be either inter-crystalline or intra-crystalline.

To summarise, coral fluorescence appears to result from a combination of yellow/orange patches and blue background fluorescence. The fluorescence appears to originate from dark, possibly organic, inclusions which could conceivably be humic in origin. Although the location of these inclusions was not absolutely determined, the larger ones, at least, are probably inter-crystalline.

5.3.2 Nature of the inclusions: Although the origin of these dark inclusions was not absolutely determined, the following possibilities are suggested:

(a) As polyp tissue is also yellow/orange in colour when viewed under a u/v lamp (see Chapter 4, Figure 4.39, page 133), the yellow/orange patches could conceivably be due to the presence of trapped polyp tissue. As emission spectra produced by a number of these yellow/orange patches are very consistent, it is possible that their compositions are similar. This would be expected if polyp tissue was incorporated into the skeleton in solid lumps. Work in Chapter 4, section 4.8.1, page 132, has shown that polyp tissue could possibly represent a highly concentrated source of humic acid or humic acid-like fluorophores. If the polyp tissue is incorporated as whole lumps into the skeleton, then the concentration of fluorophores in the incorporated tissue is not going to be diluted as was the case when polyp tissue coral solutions were made. Thus, if solid state coral fluorescence is due to inclusions of polyp tissue, one might expect all the inclusions to be yellow/orange in colour when viewed using a fluorescence microscope. The fact that they are not (i.e. sub-micron sized inclusions appear to be various shades of blue) could be because of seasonal variations in the concentration of fluorophores in the polyp tissue. Although work in Chapter 4, section 4.8.2, page 136, has shown that the concentration of fluorophores in the surrounding water varies seasonally, no visual differences were observed in the colour of polyp tissue fluorescence collected in either the

wet or the dry season. Thus, it would appear that some other mechanism must be involved in the production of the blue background fluorescence.

(b) The yellow/orange fluorescent patches could also be due to areas which, for some unknown reason, act as a focal point for the accumulation of organic matter (not all of which will contain fluorophores). Results in Chapter 4 (see section 4.6.1, page 98) have shown that as the concentration of humic acid fluorophores increased above the ET threshold, fluorescence emissions shifted to longer wavelengths. Although only a green/yellow colour was produced with a 144 ppm Fluka humic acid solution, if the concentration was increased further, a yellow/orange colour would be expected. In this scenario, the proportion of humic-like fluorophores to non-fluorescent organics (or inorganics) in the inclusion would determine the effective fluorophore concentration and therefore the colour of fluorescence. Thus, a range of fluorescent colours would be expected from blue to yellow/orange depending on the concentration of fluorophores in the inclusion. In addition, the colour of fluorescence would be independent of the size of the inclusion as colour would depend on the concentration of fluorophores to non-fluorophores in the inclusion. However, there are a number of problems associated with this model: (1) the colour of fluorescent emissions seen using a fluorescence microscope did not range from blue to yellow/orange, and (2) the colour of emissions appear to be related to the size of the inclusion i.e. small inclusions blue, large inclusions yellow/orange.

(c) There could be a critical size of inclusion below which ET can not occur. For example, in the extreme case of an inclusion consisting of one fluorophore only, significant ET could not occur as there would not be any other fluorophores to transfer the energy to. The larger the inclusion, the greater the amount of ET that is possible and the more yellow/orange the fluorescence. The fact that yellow/orange fluorescence is only observed from relatively large inclusions would support this hypothesis i.e. the concentration of fluorophores in a large patch is sufficiently high for ET to occur. ET process would be less effective in smaller inclusions and thus shorter wavelength emissions would be more dominant. Although polyp tissue retained its orange colour when crushed below 20 μm, it was not possible to say what happens at the sub-micron level. There are, however, a number of problems with this scenario: (1) it does not explain why the largest organic inclusions (i.e. the ones giving rise to the 35 μm

yellow/orange patches) were also yellow/orange and not red which would be expected if size and fluorophore concentration were positively related, and (2) it does not explain why inclusions seem to be split into two size fractions i.e. why are there no medium size inclusions that produce a green/yellow fluorescence?

(d) The fact that (1) there is no continuous range of colours from blue, where fluorophore concentrations are below the ET threshold, through greens, yellows and oranges to reds (all of which should have fluorophore concentrations above the ET threshold), and (2) that the colour and the size of the inclusion appear to be related, could suggest that the inclusion of organic material into the skeleton occurs by more than one process. One plausible explanation is that the larger inclusions (i.e. those that give rise to the yellow/orange fluorescent patches) are due to polyp tissue whereas the sub-micron sized inclusions are due to some, as yet, unidentified mechanism. Possible mechanisms are discussed in the next section.

To summarise, although the origin of the fluorescent inclusions was not absolutely identified, the fact that large patches were one colour (yellow/orange) and sub-micron sized inclusions another (blue), could suggest that the inclusion of organic material into the skeleton occurs by more than one process. Although the nature of source or sources of the fluorescence was not absolutely identified, the fact that the yellow/orange fluorescent patches were very similar in colour to polyp tissue when examined under a u/v lamp suggests that inclusions of polyp tissue could very well be an intrinsic part of coral fluorescence.

5.3.3 A possible mechanism for the incorporation of organic inclusions into the skeleton: Although various workers have already suggested that organics, and in particular humic acids, are the primary cause of coral fluorescence (Boto and Isdale 1985; Smith *et al* 1989; Klein *et al* 1990), the manner in which they are incorporated has not previously been discussed. Work in this chapter suggests that some of the organic matter thought to be responsible for coral fluorescence is incorporated as discrete patches rather than, or in addition to, individual molecules bound onto the aragonite lattice in some way. If the larger yellow/orange fluorescent patches are due to polyp tissue, then one possible mechanism for their inclusion was suggested by Constantz (1986) in his review of coral growth. He reports that growing fasciculi lift up the calicoblastic layer as they grow, and that in places, sheets of tissue sometimes become incorporated into the skeleton. Constantz also reports that these entrapped

tissue layers have a sporadic distribution which is in keeping with the apparently random distribution of the yellow/orange patches in the skeleton. The reason why more yellow/orange fluorescent patches occur in bright bands could have something to do with the rate at which polyp tissue is incorporated into the skeleton. Alternatively the rate at which polyp tissue is incorporated into the skeleton could remain more or less constant throughout the year, but the calcification rate could vary, thus controlling the ratio of yellow/orange fluorescent patches to aragonite (this is discussed in Chapter 7, section 7.3.1, page 227). As for the sub-micron sized inclusions (or even the large ones if they are not polyp tissue), a mechanism for their inclusion is not so clear. The role of organics in the coral skeleton is a highly controversial subject (see Mitterer and Cunningham 1985, for a full review). It is therefore possible that the polyp provides organic material, either actively (because it is required for skeletogenesis) or passively (as excreta), for uptake into the skeleton. Although not all of this material may contain fluorophores, it is possible that some of it does. The idea that it is unwanted material (i.e. excreta) is preferred because, if, for example, humic acids were essential in skeletogenesis, then a more even distribution of fluorophores might be expected. As the number of fluorophores in certain parts of the skeleton would appear to be very low (barely more fluorescent than the pore spaces in some cases), either fluorophores are not an essential ingredient in any so called organic matrix/template, or the molecules that they are associated with are not essential for skeletogenesis.

5.3.4 The abundance of yellow/orange fluorescent patches in the skeleton: Although yellow/orange fluorescent patches were found in both bright and dull bands, observations suggested that they were more numerous in bright bands. When the coral skeleton was examined under low magnification (this was done to enable a larger area to be examined thus giving a more reliable estimate of abundance), fluorescent intensity decreased so much that it was not possible to accurately map the distribution of these yellow/orange fluorescent patches in either band. If more time had been available, the distribution of yellow/orange fluorescent patches could have been achieved by point counting a large number of photographs taken at a magnification of x600.

5.3.5 Fluorescent banding: Possible mechanisms: Although work in Chapter 2, (see section 2.4, page 34), has shown that u/v light can penetrate solid aragonite to a depth of between 35 μm and 50 μm, much greater depths of u/v penetration have been recorded in coral skeletons (between 500 μm and 1000 μm depending on bulk

density/porosity of the sample) as a consequence of porosity, i.e. the pore spaces allow scattered/reflected light to penetrate further into the skeleton although not actually any further into the carbonate. As the thin section was only 30 μm thick, most of the fluorophores in the sample were probably excited to some degree. Therefore, in a piece of coral 500 μm thick, the number of yellow/orange fluorescent patches could be as much as 17 times greater than in the thin section, and 34 times greater for a piece of coral 1000 μm thick. Whether this would be enough to produce bright band fluorescence could depend on the ratio of yellow/orange fluorescent patches to background fluorescence. The higher the proportion of yellow/orange fluorescent patches to background fluorescence, the greater the proportion of longer wavelength emissions that will be seen by the eye and the greater the chances of fluorescent bands being a different colour. It should, however, be mentioned that our eyes do not see all colours equally (see Chapter 4, Figure 4.12, page 86, for the general eye response curve). The eye is much better at seeing yellow/orange light than it is at seeing blue light. Therefore, in a mixture of blue and yellow/orange light, the ratio of blue to yellow/orange light would have to be quite high before the blue colour is seen. Conversely, the ratio of yellow/orange to blue light would not have to be very high before the yellow/orange colour was seen. Therefore, in relation to coral fluorescence, the ratio of yellow/orange fluorescent patches to blue background fluorescence does not have to be that high for the former to make a significant effect on the colour of fluorescence seen by the eye. As bright bands appear to have more yellow/orange fluorescent patches (and therefore a higher yellow/orange to background fluorescence ratio) than dull bands, a colour difference between the two might be anticipated in the solid state.

5.4 IMPLICATIONS FOR CORAL FLUORESCENCE:

Results in this chapter have shown that there is a marked difference in the manner in which coral fluorophores are distributed in the solid and solution phases (i.e. heterogeneous versus homogeneous respectively). As a direct consequence of this, the amount of interaction that can occur between the various fluorophore patches, or, for that matter, between fluorophore patches and fluorescent quenching molecules, is likely to be considerably less than in solution where a high degree of interaction is possible. Work by Matthews et al (in review) has shown that various fluorophores can quench each other, in addition to which, work in Chapter 4, section 4.6.2, page 115, has shown that the metal ions manganese and iron can also quench both humic

acid and coral fluorescence. These quenching agents will therefore have less of an effect if they are isolated from the fluorophores, a scenario which is more likely to occur in the solid state. Thus it is conceivable that the fluorescence emitted by certain molecules which would have been quenched by humic acids and/or manganese and iron in the solution phase, may well be seen in the solid state. This, therefore, suggests that coral fluorescence is best studied in the solid state.

It is also important to remember that in the solution phase, with the pH adjusted to 9, the majority of the coral fluorophores (i.e. humic acids) will be in solution. Thus, regardless of whether the yellow/orange fluorescent patches are due to polyp tissue or to high concentration humic acid inclusions, when either of coral fluorophores or humic acids are in solution, fluorescent emissions shift to shorter wavelengths (i.e. from orange to blue). Assuming that the yellow/orange patches are due to humic acids, when the coral skeleton is dissolved, the fluorophores will no longer exist in a concentrated organic patch (i.e. they will dissolve and diffuse evenly throughout the solution). Thus, only in the solid state are such high concentration fluorophore patches likely to exist. This is, therefore, a very strong argument for studying coral fluorescence in the solid state. Although the large inclusions are certainly a different colour to the background fluorescence when viewed using a fluorescence microscope, it is not known for certain whether there are enough of them to have a significant effect on the colour of fluorescence seen by the eye. This subject is discussed in Chapter 6, section 6.4, page 201.

5.5 CONCLUSIONS:

(A) Coral fluorescence, when examined using a fluorescence microscope, appears to consist of two main elements, yellow/orange fluorescent patches and mottled blue background fluorescence. This would suggest that the distribution of fluorophores in the skeleton is heterogeneous.

(B) Although it was not possible to absolutely determine what the yellow/orange fluorescent patches were, their dark brown/black appearance in transmitted light and the fact that their emission spectra are very similar to that of humic acid suggests that they are organic in origin. One possible source for the yellow/orange patches is polyp tissue which fluoresces with a similar colour when excited by u/v light, a feature thought to be due to a high concentration of humic acids.

(C) Although a mechanism for the incorporation of these organic inclusions into the skeleton was not absolutely determined, polyp tissue could be trapped between growing fasciculi and the smaller inclusions could be incorporated because of their role in skeletogenesis.

(D) The fluorescence seen in solid state corals is a complex mixture of light that originates from a number of isolated sources within the skeleton. The fluorescence from these sources has minimal effect on the fluorescence from other areas (i.e. they can act in isolation) which therefore allows the fluorescence from fluorophores which would have been quenched in solution to be seen.

(E) While areas of high fluorophore concentration can exist in the solid state (i.e. the yellow/orange fluorescent patches), they are unlikely to exist in the solution phase. This is because, when coral skeleton is dissolved with HCl and the resulting solution adjusted to pH 9, the vast majority of the fluorophores (humic acids) will not only be in solution, but evenly distributed throughout that solution. This, and the dilution factor involved when making coral solutions (between 10 and 30), is probably the reason why coral solution fluorescence is, in general, below the ET threshold and colours other than blue are not seen. Thus, coral fluorescence should be examined in the solid state.

(F) Fluorescent banding as a colour phenomenon, but possibly also as an intensity phenomenon (or even both), could be related to the concentration of micron-scale organic inclusions, with bright bands having more inclusions than dull bands.

CHAPTER 6. MEASURING SOLID STATE CORAL FLUORESCENCE

CHAPTER 6. MEASURING SOLID STATE CORAL FLUORESCENCE

6.1 INTRODUCTION:

In order to effectively compare coral fluorescence with environmental data and identify potential cyclic patterns of fluorescence, a long record of coral fluorescence is required. Although such a record could be obtained from coral solutions in which the calcium carbonate had been dissolved, it would not only take a lot of time to obtain and also destroy the coral skeleton, but once released from the skeleton, the fluorophores would decay quite rapidly even if kept in the dark. Investigation of coral fluorescence in the solid state is a quick, non-destructive process and at present appears to be the only way of detecting differences in terms of the colour of fluorescence between bright and dull bands. Thus, the objective of the work described in this chapter was to develop a technique for measuring fluorescence from solid coral cores. By doing this it may be possible to compare coral fluorescence with environmental parameters such as rainfall, solar radiation and productivity.

6.2 SOLID STATE EXCITATION/EMISSION SPECTRA OBTAINED USING THE LS-5 SPECTROFLUORIMETER:

Preliminary experiments were undertaken to explore the possibility of using the LS-5 spectrofluorimeter to produce excitation/emission (EXEM) spectra directly from solid coral.

6.2.1 Methods and materials: A slice of coral skeleton was cut to fit exactly inside a cuvette and positioned in such a way that the excitation beam (10 mm by 2 mm) excited one band only. The angle at which the coral sample was presented to the u/v beam was adjusted until an acceptable intensity reading was obtained. This step was required because the maximum fluorescent intensity was often too large to be recorded by the LS-5. As fluorescence was measured from the centre of the cuvette, the coral skeleton was positioned as shown in Figure 6.1. Thus, unlike solution phase spectroscopy, fluorescence generated at point, 'f', does not interact with other molecules on its journey to the photomultiplier (PMT).

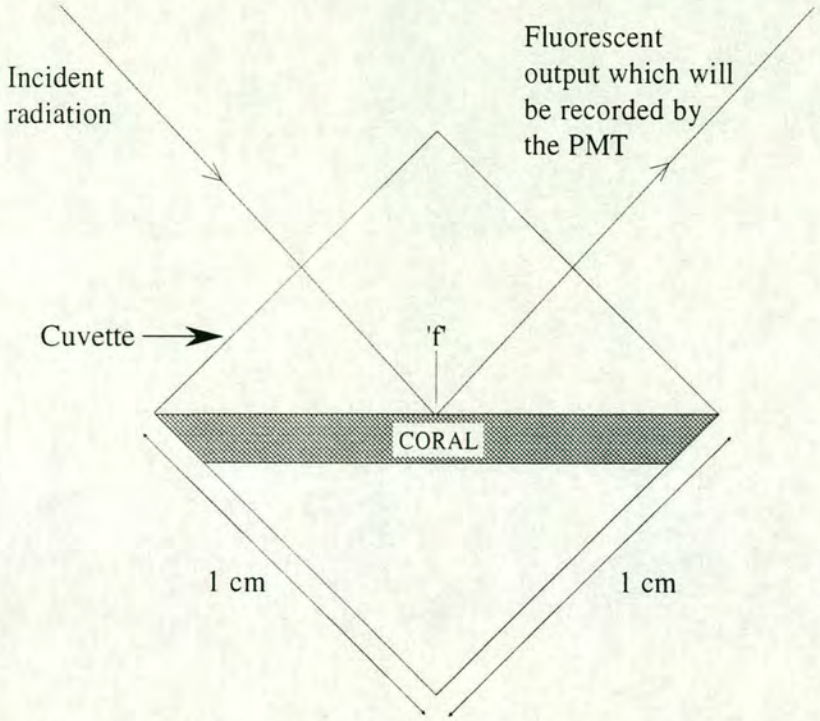


Figure 6.1 Schematic diagram showing the angle of the coral skeleton relative to the incident radiation.

6.2.2 Results and discussion: Before the results are discussed, it should be pointed out that work in Chapter 2 (see Table 2.3 on page 34) has demonstrated that the depth of u/v penetration into aragonite depends on the wavelength of u/v light used. Results showed that the depth of u/v penetration increased with increasing wavelength and therefore, the volume of skeleton excited will change. Thus, the intensities of each fluorophore group should not be used as an estimate of the concentration of each type of fluorophore present as different volumes are being excited. Because the intensity of fluorescence was so strongly dependent on the angle of the coral to the excitation beam, reproducibility was practically zero. Although various modifications were tried, for example: (1) a specially adapted holder was used which held the cuvette in exactly the same place, and (2) coral powder was used to remove the porosity effect, reproducibility was still poor. It was therefore not possible to determine whether bright band fluorescence was more intense that dull band fluorescence. Bearing this in mind, the shape of solid coral EXEM spectra were similar to coral solution EXEM spectra although the former had a more pronounced 390 nm excitation peak (possibly due to higher fluorophore concentrations), and a lot more Rayleigh-Tyndall scattering. A feature common to all solid coral EXEM spectra was an excitation trough at 300 nm (see Figure 6.2).

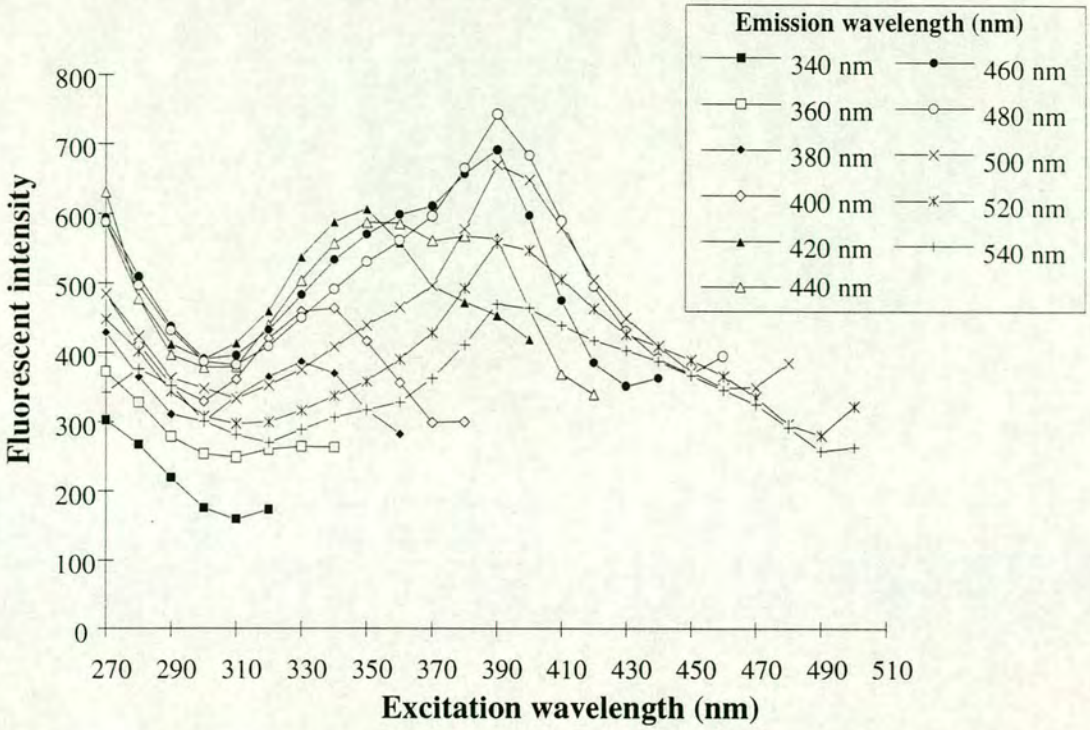


Figure 6.2 Solid EXEM spectra. Coral showing the characteristic 300 nm excitation wavelength dip.

This dip was thought to be due to aragonite absorption and appeared to reduce the intensity of the 330-340 nm excitation peak. In order to understand what effect $CaCO_3$ absorption has on solid coral EXEM spectra, its absorption characteristics had to be determined. Alas, it was not possible to determine the absorption characteristics of pure $CaCO_3$ either in solution (virtually insoluble) or in the solid state (because no pure aragonite was available). Therefore, the absorption characteristics of $CaCl_2$ and $Ca(NO_3)_2$ were examined. $CaCl_2$ was used to see if the 300 nm excitation trough was associated with Ca^{2+} while $Ca(NO_3)_2$ was used to see if the 300 nm excitation trough was associated with the nitrate structure (the nitrate structure being very similar to that of carbonate). Results showed that while $CaCl_2$ did not have an absorption peak at 300 nm, $Ca(NO_3)_2$ did (see Figure 6.3). Therefore, it is possible, but not certain, that carbonate would have a similar effect.

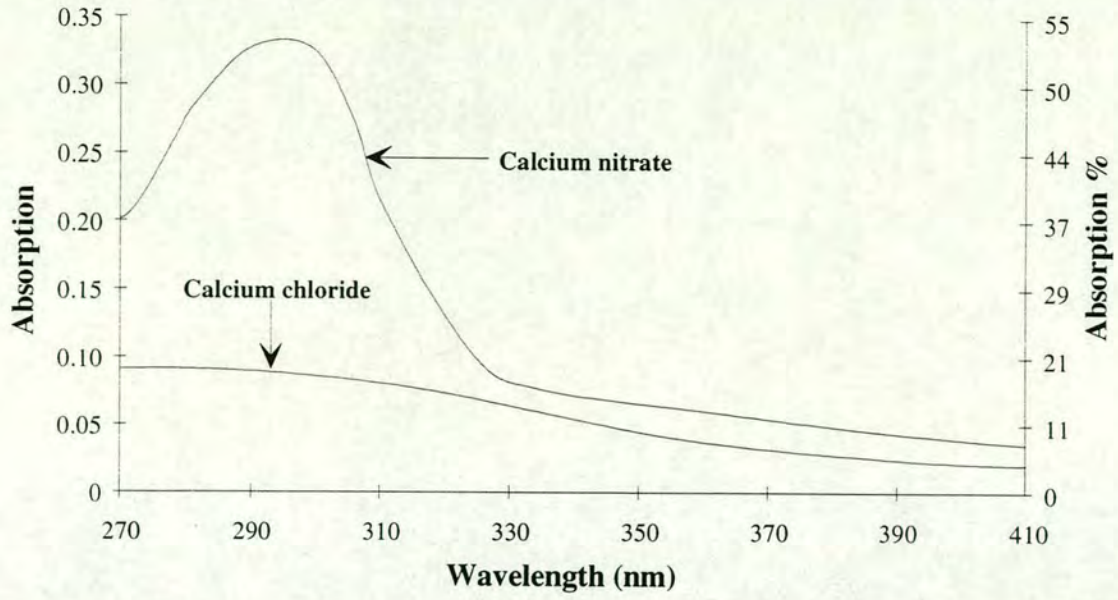


Figure 6.3 Absorption spectra of 1000 ppm solutions of (a) $CaCl_2$ and (b) $Ca(NO_3)_2$.

The high fluorescent intensities in solid coral EXEM spectra were originally thought to be due to scattered/reflected light that might be superimposed on top of the coral fluorescent signal. As shown in Figure 6.1 on page 170, plenty of reflected light might be expected to reach the PMT as the reflected path is the same as the fluorescence detection path of the PMT. In order to estimate the amount of light scattered/reflected from a solid coral surface, the LS-5 was set to record emissions at 580 nm while the excitation wavelength varied between 400 nm and 570 nm, and 590 nm and 610 nm. The range 570-590 nm was omitted as the amount of scattered/reflected light produced in this region could potentially damage the PMT. With the LS-5 set to record emissions at 580 nm, the amount of fluorescence recorded should be insignificant (due in part to PMT response). This, however, should not affect the LS-5's ability to record scattered/reflected light as the amount of scattered/reflected light at any excitation wavelength should considerably outweigh the amount of fluorescence generated at that wavelength. Results showed that for an emission wavelength of 580 nm, scattered/reflected light from the solid coral surface was insignificant for excitation wavelengths between 400 nm and 550 nm and above 610 nm (see Figure 6.4). In other words, scattered/reflected light will only significantly affect the last 30 nm of any emission line. These results therefore, strongly suggested that the high fluorescent intensities were real i.e. due to coral fluorescence.

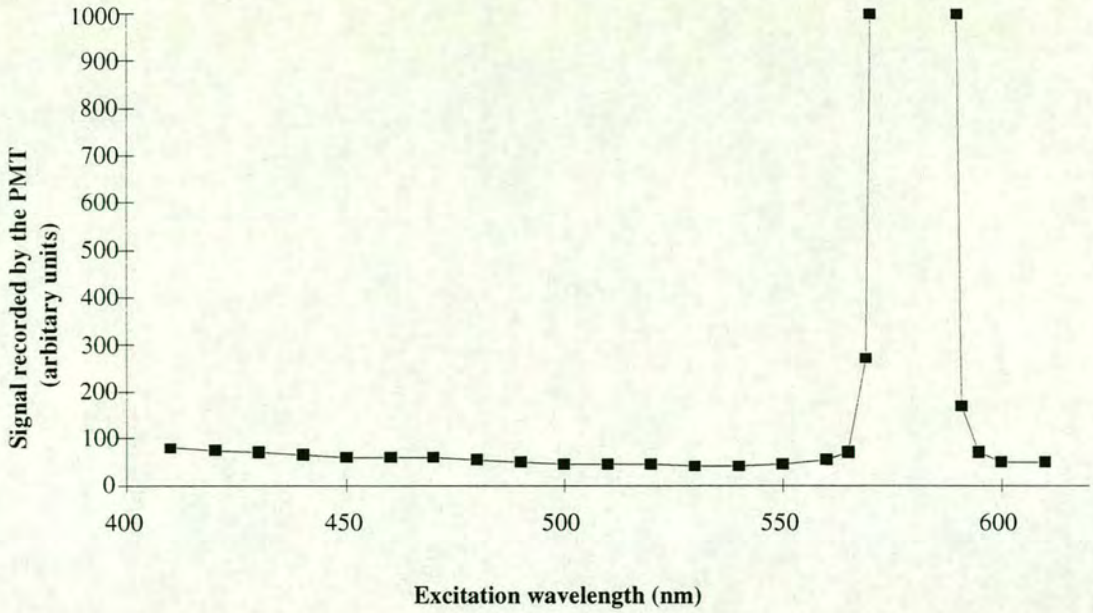


Figure 6.4 Scattered/reflected light from a solid coral (powder) surface.

Three explanations are proposed to account for the increased intensity of fluorescent emissions from solid coral skeletons:

(a) The most likely reason for the difference in fluorescent intensities between solid and solution phases is that the concentration of fluorophores in the solid state is higher than in coral solutions. In Chapter 4 (see section 4.6.1, page 110), the concentration of fluorophores in 1M coral solutions was calculated to be between 10 and 30 times lower than in the solid state.

(b) The amount of incident light absorbed could also vary. Most of the exciting light passes straight through coral solutions whereas results in Chapter 2 (see Table 2.3 on page 35), suggests that very little u/v light passes straight through coral skeletons. Even though not all of this light is absorbed (some is undoubtedly scattered/reflected off the coral surface), the porous nature of the skeleton will result in much of the scattered/reflected light being absorbed in other parts of the skeleton that were not excited directly by the incident radiation. Although $CaCO_3$ does not fluoresce itself, it may transfer some of the energy it absorbs on to the fluorophores which results in an increase in fluorescent efficiency.

(c) The fluorescence emitted from solid coral does not interact with other molecules on its way out of the cuvette because of the way the coral is

positioned (see Figure 6.1 on page 170). In the solution phase, however, the fluorescence emitted can interact with other molecules before leaving the cuvette as the solution fills the entire cuvette. Although the effects of this were not determined, energy (and therefore intensity) is likely to be lost with every interaction. The amount of interactions and the nature of those interactions will determine the amount of energy lost in this way.

The fact that there is a dip at 300 nm gives the impression that there is an excitation peak at 270-280 nm. Although the 270-280 nm excitation peak could be used as evidence for the presence of either isoxanthopterin or tryptophan in coral skeletons, it could also be an artefact of this dip. Perhaps this dip (possibly due to aragonite absorption) was the reason why Boto and Isdale (1985) noticed a shift to longer wavelength emissions when solid coral skeleton was dissolved with HCl to make solutions. Thus, it is conceivable that the higher the aragonite to fluorophore ratio, the greater the dip and the more the 330-340 nm excitation peak will be depressed thus giving the impression of a shift towards longer emission wavelengths.

6.2.3 Summary/Key points:

(1) Bright and dull band EXEM spectra were very similar in terms of shape, with characteristic humic acid excitation peaks at 330-345 nm and 390 nm. However, the inability to obtain reproducible data meant that it was not possible to compare bright and dull band intensities.

(2) The dominance of the 390 nm excitation peak in solid state EXEM spectra could be attributed to CO_3^{2-} absorption which reduces the size of the 330-345 nm excitation peak and cause the excitation dip at 300 nm.

(3) The high fluorescent intensities recorded for solid state coral skeleton could be due to the concentration of fluorophores being much higher than in coral solutions. In addition, absorption and energy transfer by aragonite to coral fluorophores could also play a part.

Even though the LS-5 spectrofluorimeter could produce EXEM spectra, it was considered to be unsuitable for the following reasons:

(a) The position of the solid coral slab relative to the incident light was crucial in determining fluorescent intensity. As this position could not be fixed, results were exceedingly variable.

(b) One of the aims of this chapter was to record the fluorescent characteristics of a large number of bands from cores which were often over a meter in length. The LS-5 spectrofluorimeter could not therefore be used as it could not accommodate samples which were longer than 10 cm.

Thus, a purpose-built fluorescence measuring device had to be developed which could generate highly reproducible results from solid coral cores of approximately one meter in length (see section 6.3).

6.3 THE FLUORESCENCE MEASURING DEVICE:

6.3.1 Coral preparation: Coral cores, collected by Tudhope and Scoffin, were used in this chapter because they contain a large number of fluorescent couplets which would hopefully enable a long record of fluorescence to be obtained. For basic coral preparation see Chapter 1, section 1.5, pages 15-21. As porosity has been shown to affect fluorescent intensity (see Chapter 2), variations in the orientation of the corallite could introduce unwanted noise into the results. In an attempt to overcome this problem, black epoxy resin (supplied by B and K resins) was impregnated into the pore spaces under vacuum (see Figure 6.5).

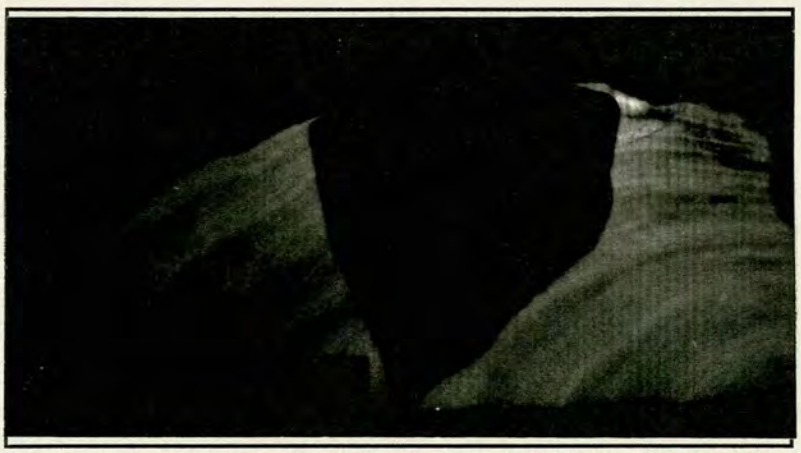


Figure 6.5 The effect that impregnating coral skeleton (PB-3-90) with dyed black epoxy-resin has on coral fluorescence. Central section was impregnated with black epoxy resin. Scale bar 5 cm. As can be seen, very little fluorescence is emitted from the section containing the epoxy resin.

The coral was then ground down to reveal the uppermost part of the coral skeleton. However, the amount of fluorescence recorded by the PMT was so low that background noise became a considerable problem. For this reason, the corals were not impregnated. Thus, in order to minimise the effects of porosity/corallite orientation, coral cores were cut along the axis of maximum growth. However, because this axis often varied over quite short distances, perfect longitudinal section were rarely produced. The coral was cut to a thickness of 10 mm as observations have demonstrated that while fluorescent intensity is constant to the eye at thicknesses greater than ~1 mm, fluorescent intensity decreases when the skeleton is thinner than this (see Figure 6.6).

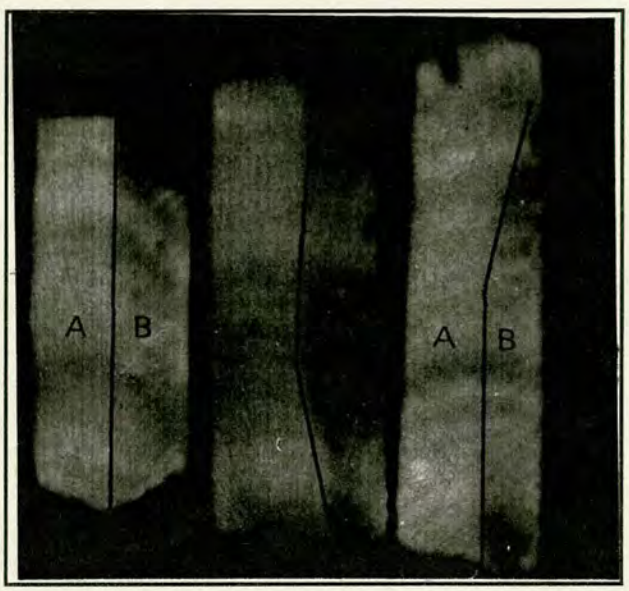


Figure 6.6 The effect the thickness of a coral block has on coral fluorescence. Coral blocks were cut such that one half was approximately 1 mm thick and the other half was approximately 500 μm thick. Side (A) = ~1 mm thick, and side (B) = ~500 μm thick. It can be seen that the thicker side is brighter, a phenomenon that probably arises because more fluorophores are excited in the thicker half. Once the thickness of the coral block has exceeded ~1 mm, no additional fluorophores are excited. This is probably because the u/v light can not penetrate the skeleton any further.

The cores were polished to remove grooves and ridges which have also been shown to affect fluorescent intensity (see Chapter 2, Figure 2.5, page 33). Due to the porous nature of the coral skeleton, carborundum grit was not used for the following reasons: (1) it was difficult to remove from the skeleton even after extensive sonicating, and (2) once incorporated into the skeleton, it inhibited fluorescence (probably because it was black in colour). Thus, the coral skeleton was ground by hand using first 200 and then 600 grade carborundum paper until a smooth finish was produced. The fluorescent profiles presented in this chapter were taken from coral sections in which the fluorescent bands were clearly discernible. This was done to enable a fluorescent couplet to be easily identified so that the coral could be divided into yearly sections thus allowing the fluorescent data to be compared with environmental records.

6.3.2 The fluorescence measuring device: The apparatus used in this chapter is show in Figure 6.7. A u/v source was focused onto the surface of the coral core using a fused quartz lens. Using a series of plano-convex lenses, fluorescent emissions were focused on to a PMT which recorded the signal as a voltage. A u/v cut-off filter was also employed to prevent u/v radiation from the lamp being recorded by the PMT.

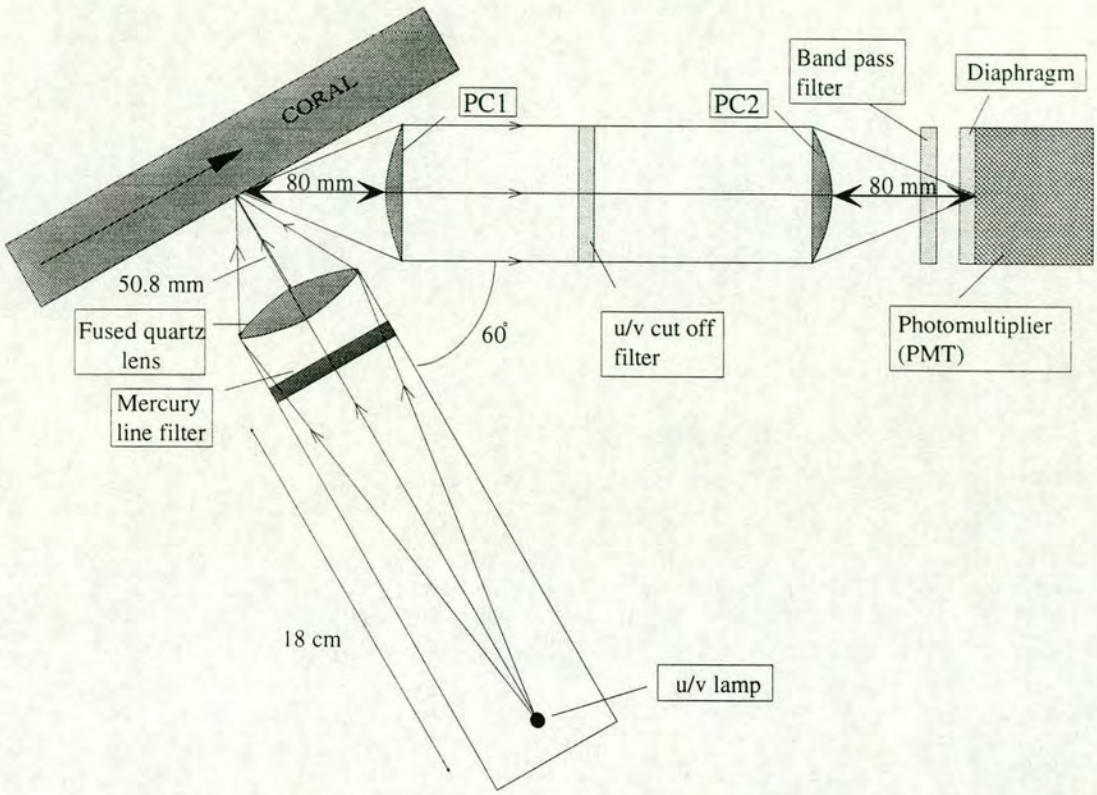


Figure 6.7 The fluorescence measuring device.

Before discussing the individual components that make up the fluorescence measuring device, it must be pointed out that although Boto and Isdale (1985) used a more complicated device for measuring solid state fluorescence, it was decided in the interests of cost, that a low budget prototype should be developed first. If this proved a success then upgraded components would be introduced.

The excitation source: A mercury-argon lamp (model 6035 Hg(A) supplied by Oriel Corporation) was used to excite the coral core. Although a two minute warm-up period was required for wavelength stability, it took thirty minutes for emission intensity to stabilise. Thus, no fluorescent measurements were taken during this

period. Although the lamp generated radiation over a wide range of wavelengths, it did not emit all wavelengths equally, with approximately 90% of the output being concentrated at 254 nm. The lamp does, however, emit considerable amounts of radiation in the visible part of the spectrum which could be recorded along with coral fluorescence. In order to prevent this from happening, a short wave filter was placed in front of the lamp (Model 6041 supplied by Oriel Corporation). Although this filter was designed to cut out all visible light, up to 10% is transmitted between 400-420 nm and 30% between 670-700 nm (no light is transmitted between 430-670 nm). As fluorescent emissions were recorded at 450 nm, 500 nm and 550 nm, this was not considered to be a problem. The short-wave filter also reduces the amount of u/v radiation transmitted by ~50% at 254 nm and ~15% at 365 nm.

Bandpass filters: Bandpass filters transmit light within a defined spectral range and were used (1) to control the wavelength of the excitation beam, and (2) to measure the fluorescence emitted at specific wavelengths from the coral. The filters used in this chapter were designed for operation at 23°C, and deviations from this value in the range -60°C to +60°C will produce peak wavelength shifts approximately linear with temperature. The exact shift depends on the filter concerned but typically these range between 0.02 and 0.03 nm per °C between 400 nm and 650 nm. Care should be taken not to expose filters to temperatures outside the -60 to +60°C range as permanent alteration of the filters performance may result. The bandpass filters used in this chapter (supplied by Ealing Electro-Optics) have been divided into two groups:

(a) **Excitation bandpass filters**: In this chapter, coral cores were excited at either 254 nm or 365 nm. The 365 nm bandpass filter was chosen because coral fluorescence studies have traditionally used this wavelength. The 254 nm bandpass filter was chosen for two reasons: (1) to see what effect shorter wave u/v had on solid coral fluorescence, and (2) because it corresponded with the main emission peak of the mercury-argon lamp. Each bandpass filter has an associated bandwidth figure which represents the spectral width of wavelengths transmitted. This was 7 nm for the 254 nm bandpass filter and 10.7 nm for the 365 nm bandpass filter (i.e. the excitation beam was tightly constrained). In addition, each bandpass filter has an associated peak transmission. This relates to the amount of light transmitted within the bandwidth as a percentage of the total amount of light supplied within that bandwidth. This figure is approximately 10% for the 254 nm bandpass filter and 38% for the 365 nm

bandpass filter. In other words, the 254 nm bandpass filter only transmits 10% of the radiation between 250.5 nm and 257.5 nm, while the 365 nm bandpass filter only transmits 38% of the radiation between 360 nm and 370 nm (and essentially nothing outside this range). Because of the differences in peak transmission and band width, 254 nm and 365 nm fluorescent profiles should not be directly compared unless a correction is applied. This correction factor would also have to take into consideration the fact that 254 nm u/v light is unlikely to penetrate the aragonite as far as 365 nm u/v light (see Chapter 2, Table 2.3, page 34).

(b) **Emission bandpass filters**: Coral fluorescence was measured using the following bandpass filters: 450 nm, 500 nm and 550 nm. A larger number of filters or even a monochromator (a device used for isolating narrow segments of spectral radiation from a broader range of wavelengths) would have been used if more time was available as initial results were very encouraging (discussed later in section 6.3.4, pages 189-200). Although each emission bandpass filter had a bandwidth of 40 nm which meant that fluorescent emissions were measured over quite a wide range of wavelengths, fluorescence was attributed to the peak transmission wavelength of the bandpass filter. As the peak transmission value for each of the emission bandpass filters used in this chapter was the same (50%), fluorescent emissions from the coral did not have to be adjusted to account for variations in bandpass filter transmission.

The Photomultiplier: The PMT used to measure solid coral fluorescence in this study was the R928 which is part of the H957-08 side-on PMT assembly supplied by Hamamatsu. When radiation enters the PMT (see Figure 6.8), the photo-cathode emits photoelectrons into the vacuum. These photoelectrons are then directed by the focusing electrode voltages towards the electron multiplier where the electrons are multiplied by the process of secondary emission. The electrons produced in this way are then collected by the anode.

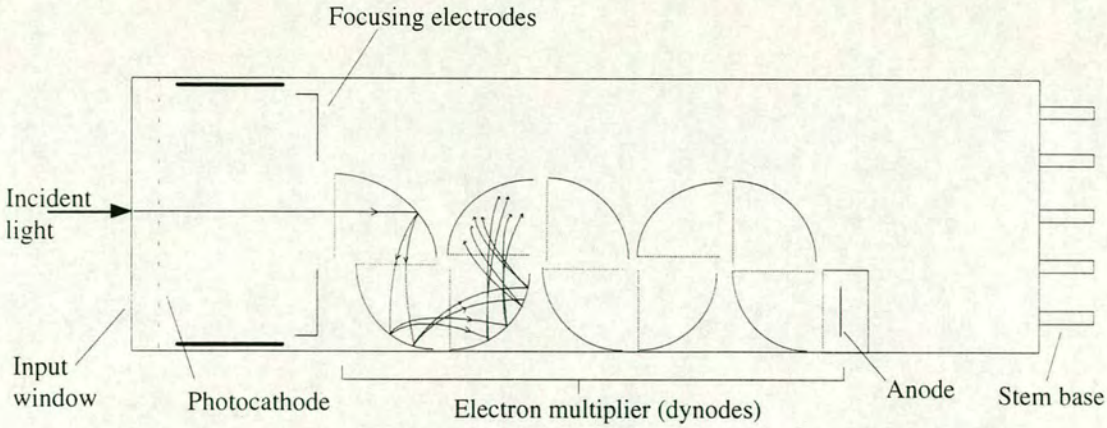


Figure 6.8 Although this is a cross section of a head-on type PMT, the principle is the same for a side-on type PMT.

An amplifier (model C2719 supplied by Hamamatsu) was connected to the PMT which converted PMT output current to a voltage signal and thus enabled measurements of fluorescence to be made using a voltmeter. The amplifier could be adjusted to one of three sensitivity settings; low, medium and high depending on the amount of fluorescence recorded by the PMT. Although the R928 PMT records radiation in the range 185-900 nm, its spectral response depends on the wavelength of incident radiation (see Figure 6.9).

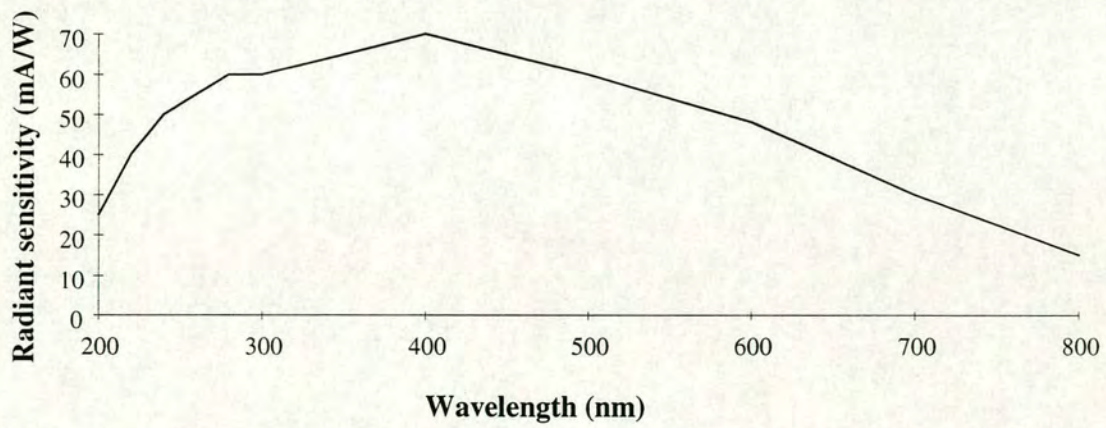


Figure 6.9 Spectral response of the R928 PMT.

Figure 6.9 shows that the R928 PMT does not record all wavelengths equally with a peak wavelength response at 400 nm. Therefore a correction factor must be applied if the intensities of fluorescence emitted at 450, 500 and 550 nm are to be directly compared.

Lenses: These were required to focus the excitation beam onto the coral surface and to direct the coral fluorescence towards the PMT. A biconvex lens (34-3020 supplied by Ealing Electro-Optics) made of fused quartz was used to focus the incident radiation from the lamp onto the surface of the coral. This was because, unlike glass which cuts out u/v radiation below approximately 360 nm, fused quartz has very good u/v transmission characteristics. Two plano-convex lenses (30-7157 supplied by Ealing Electro-optics) were used to collect the fluorescence emitted from the coral surface (PC 1) and focus it on the PMT (PC 2). Although the distance between PC 1 and PC 2 was not critical, the distances between the coral surface and PC 1, and PC 2 and the PMT were fixed at 80 mm (the focal length of each plano-convex lens).

Background noise: Background noise was attributed to the following:

(a) Radiation detected by the PMT other than coral fluorescence. Although the fluorescence measuring device was operated in a specially darkened room, a large amount of background noise was detected when the excitation and emission sections of the fluorescence measuring device were not isolated. This was attributed to u/v radiation from the lamp reaching the PMT directly. In order to reduce background noise, the excitation and the emission sections of the fluorescence measuring device were placed in separate boxes whose interiors were painted black. The PMT was isolated from the other components in the emission section by means of a blackened partition with a diaphragm fitted into it. The diaphragm could be manually adjusted thus allowing the amount of radiation reaching the PMT to be controlled.

(b) Dark current noise. Dark current noise is an inherent PMT problem and arises because a small amount of current flows in the PMT even when the tube is operated in complete darkness. The higher the supply voltage, the larger the dark current.

The clamping device and stepper motor attachments: In order to reproducibly measure coral fluorescence, it was necessary to pass the core along a known path at a fixed speed and at a certain angle. This was achieved using a clamping device which was moved in a horizontal plane by a stepper motor. Work in section 6.2 has shown that the angle the coral was presented to the u/v beam was critical in determining the intensity of the fluorescence detected. For this reason, coral core sections were

carefully positioned in the clamping device as shown in Figure 6.10 with the polished surface facing the u/v beam.

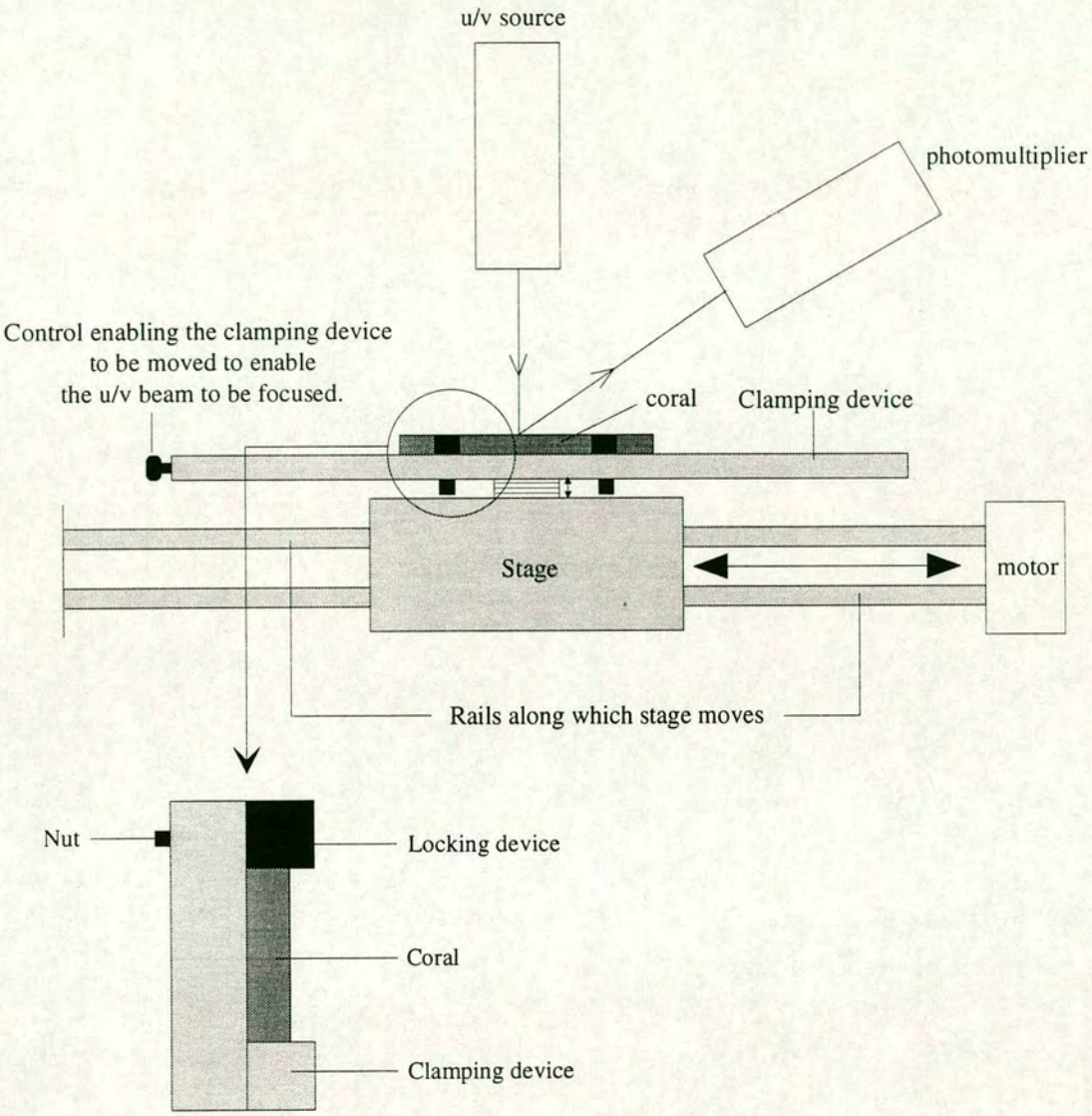


Figure 6.10 Schematic cartoon of clamping device, stage and stepper motor.

By lowering and raising the clamping device a number of different fluorescent profiles could be obtained from one coral core. The clamping device could also be moved backwards and forwards to help focus the excitation beam on to the coral surface.

6.3.3 Operational conditions:

Amplifier setting: Figure 6.11 shows that PMT sensitivity increases as the supply voltage increases (up to a maximum of 1250 v).

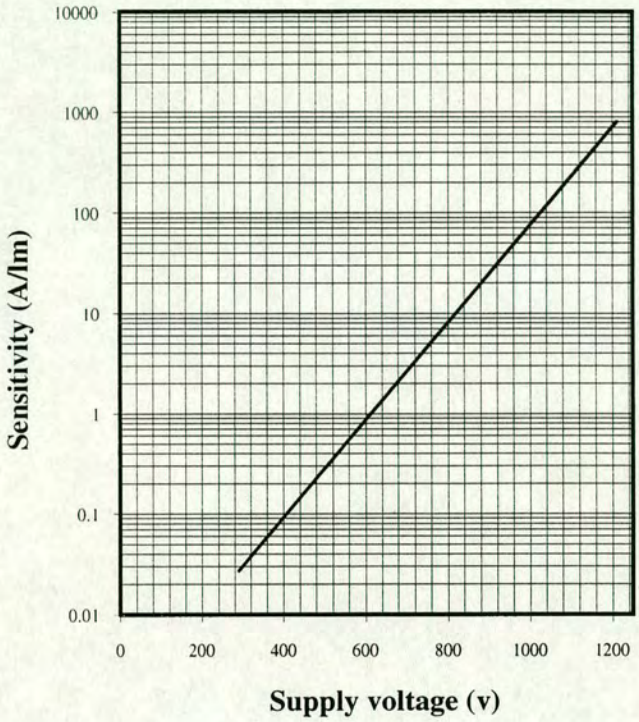


Figure 6.11 The relationship between power supply and PMT detection. Data supplied by Hamamatsu.

However, as PMT sensitivity increases, background noise also increases. Measurements in this chapter were taken with the amplifier on its medium sensitivity setting and the diaphragm in front of the PMT half closed for the following reasons:

(a) Although a very large signal was recorded when the amplifier was on its high sensitivity setting, background noise was also high and variable. Even when the size of the aperture in front of the PMT was reduced, significant amounts of background noise were still recorded by the PMT.

(b) With the amplifier on its low sensitivity setting, virtually no fluorescence was recorded.

Operating voltage: In order to determine the optimum PMT supply voltage that maximised fluorescence and minimised background noise, coral skeleton was excited at 365 nm and emissions were measured as the supply voltage was varied between 100 and 1000 volts with the amplifier on the medium sensitivity setting. A PMT supply voltage of 800 v was chosen because (1) the fluorescent output was greatly reduced when lower supply voltages were used and (2) background noise was essentially zero (see Figure 6.12). Thus, at this voltage, anything recorded by the PMT should be due to coral fluorescence.

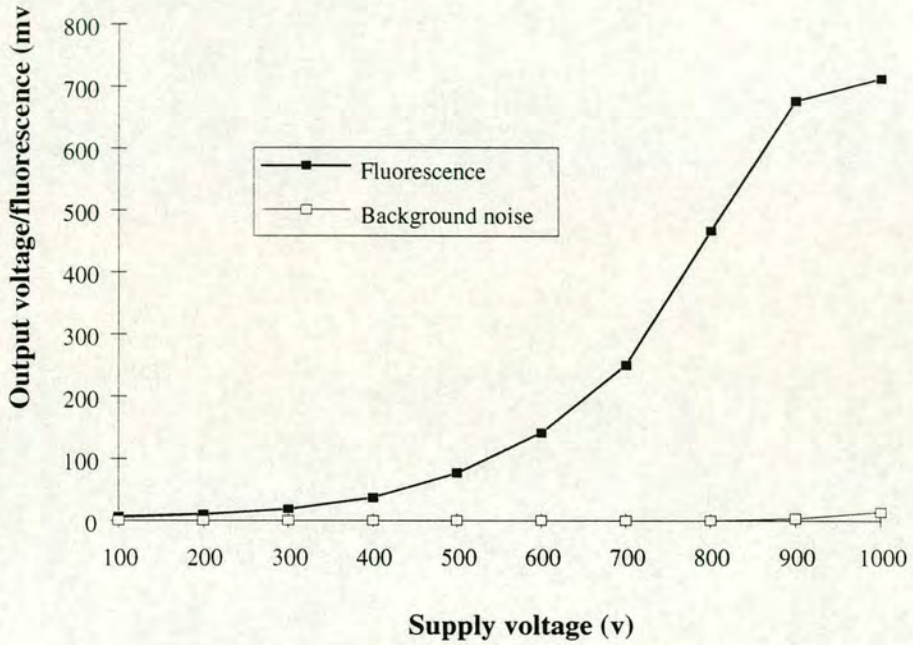


Figure 6.12 The effect of changing the supply voltage of the PMT on the fluorescent signal generated. The readings were taken using the 365 nm excitation bandpass filter and the 550 nm emission bandpass filter. Similar results were also obtained using the 450 nm and 500 nm emission bandpass filters.

The position of the lamp, the PMT and the coral: Tests were carried out in which the angle between the lamp and the coral surface was varied between 30° and 90°. The PMT was then moved into a position which maximised PMT response. Results (see Appendix A6.1) showed that irrespective of the angle between the lamp and the coral surface, the maximum amount of fluorescence was recorded when the angle between the lamp and the PMT was approximately 60°. Thus, in all subsequent work, the angle between the lamp and the PMT was fixed at 60°. The angle between the lamp and the coral surface was fixed at 90° as this enabled the excitation beam to be easily focused. An additional advantage of having the lamp at 90° to the coral surface was that it reduced the amount of reflected u/v radiation from the lamp

reaching the emission section of the fluorescence measuring device (i.e. most was reflected back towards the lamp). Some u/v light from the lamp will, however, inevitably reach the emission section of the fluorescence measuring device as a consequence of scattering, and could therefore be recorded by the PMT along with fluorescent emissions from the coral. In order to reduce the amount of u/v light recorded in this way, a u/v cut-off filter (product number 26-4697 supplied by Ealing Electro-optics plc) was incorporated into the emission section of the fluorescence measuring device (see Figure 6.7 on page 177). Although the u/v cut-off filter was supposed to cut out all u/v light, transmission spectra showed that it did allow some u/v light above 365 nm to pass through (see Figure 6.13). Thus, when coral skeleton is excited at 365 nm, a small amount of u/v light from the lamp could potentially reach the PMT. However, with the appropriate emission bandpass filter in place, the amount of u/v source light reaching the PMT should be insignificant.

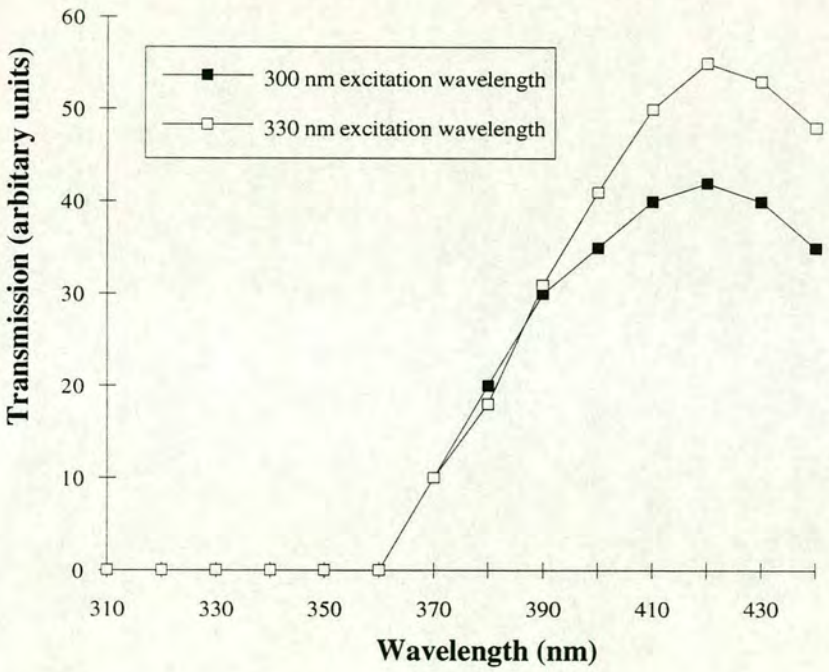


Figure 6.13 u/v cut-off filter transmission. This was determined using the LS-5 spectrofluorimeter used in Chapter 4. A coral solution was excited at two fixed wavelengths, 300 nm and 330 nm, and emissions were measured from 310 nm to 440 nm and from 340 nm to 440 nm respectively. All fluorescence recorded by the PMT passed through the u/v cut-off filter.

The size and intensity of the excitation strip: An excitation strip rather than a spot was produced because the u/v lamp was not a point source of radiation. The size and intensity of the excitation strip could be controlled by changing the position of the lamp relative to the fused quartz lens and/or by adjusting the position of the fused quartz lens relative to the coral surface. A small but intense excitation strip (5 mm

by 2 mm) was produced when the distance between the fused quartz lens and the coral was 50.8 mm and the distance between the fused quartz lens and the lamp was 18 cm. Increasing the size of the excitation strip from 5 mm by 2 mm to 20 mm by 8 mm resulted in a decrease in the intensity of the excitation strip as the same amount of incident radiation was being spread over a larger area. It also resulted in an increase in the amount of fluorescence recorded by the PMT which probably reflects an increase in the number of fluorophores excited (see Appendix A6.2). This suggested that when the excitation strip was less than 20 mm by 8 mm, coral fluorescence was not limited by the availability of u/v radiation. Although a large fluorescent signal was recorded by the PMT when the excitation area was 20 mm by 8 mm, with fluorescent band couplets being between 10-30 mm wide, detailed intra-band fluorescent analysis would not be possible. Thus, the 5 mm by 2 mm excitation strip, with the 5 mm dimension oriented along the band, was used as it generated sufficient amounts of fluorescence and enabled numerous intra-band measurements to be made.

Exposure to u/v radiation: Kouassi and Zika (1990) noticed that the fluorescence of humic substances changed when exposed to u/v radiation. They discovered that fluorescence increased through time when exposed to 265.2 nm, 280.4 nm, 296.7 nm and 313 nm u/v radiation and decreased when exposed to 334.1 nm and 366 nm u/v radiation. As the rate of change was very rapid with large increases and decreases in fluorescence noticed within two hours, the length of time the coral skeleton should be exposed to u/v radiation had to be determined. Two experiments were performed using a coral collected from Tin Smelter Bay. In one, the coral was excited with 254 nm u/v radiation for 30 minutes and in the other it was excited with 365 nm u/v radiation for 30 minutes. Because the u/v radiation may affect the fluorescent properties of the coral, different areas within the same fluorescent band were chosen.

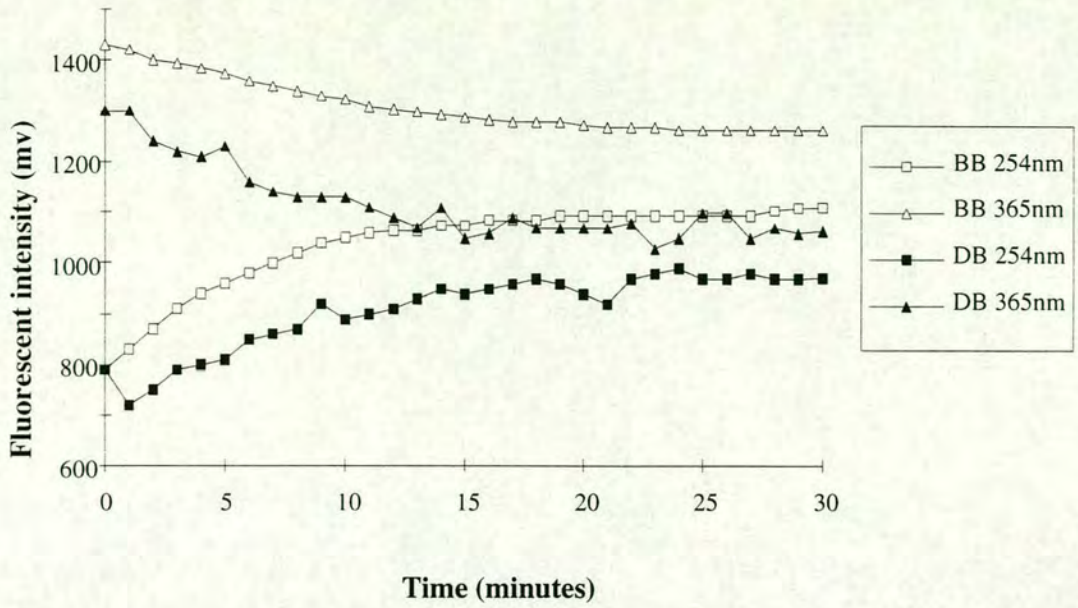


Figure 6.14 Exposure of coral to 254 nm and 365 nm u/v radiation for 30 minutes. Fluorescence recorded with the 450 nm bandpass filter.

Results show that a greater amount of fluorescence at 450 nm was generated when the coral was excited at 365 nm rather than 254 nm (see Figure 6.14). Although the reason for this was not absolutely identified, a number of possible explanations are suggested:

(a) The majority of the fluorescence generated when the coral skeleton is excited at 254 nm is probably emitted in the u/v part of the electromagnetic spectrum and is therefore not seen at longer wavelengths (i.e. in the visible part of the spectrum where fluorescent emissions are measured). However, when the coral skeleton is excited at 365 nm, the majority of the fluorescence is probably generated in the visible part of the electromagnetic spectrum which is why greater values are recorded using the 365 nm and 450 nm excitation and emission bandpass filters than the 254 nm and 450 nm excitation and emission bandpass filters.

(b) Work in Chapter 2 (see Table 2.3 on page 34) has shown that the ability of u/v light to penetrate solid aragonite increases with increasing wavelength between 300 nm and 390 nm. Thus, it is conceivable that 365 nm u/v light excited a greater number of fluorophores than 254 nm u/v light.

(c) Coble *et al* (1993) noticed that humic acids had two main excitation peaks, one at 230 nm and the other at 330-345 nm. In addition to this, work in Chapter 4, (see Figure 4.6 on page 79) has shown that a humic acid excitation peak also exists at 390 nm. Thus, a greater amount of fluorescence could be generated when the coral is excited at 365 nm because of its proximity to the 390 nm excitation peak group (exciting at 254 nm would not excite the 230 nm excitation peak fluorophores and is a long way from the 330-345 nm excitation peak fluorophores).

(d) Of course the differences could also have been due to the amount of 254 nm and 365 nm light reaching the coral surface. However, this was not thought to be a significant factor here, as crude calculations suggested that there was between 4 and 5 times more 254 nm u/v light than 365 nm u/v light reaching the coral surface (see Appendix A6.3).

Results also show that irradiation with 254 nm resulted in increases in fluorescent intensity with time while irradiation with 365 nm resulted in decreases in fluorescent intensity, thus confirming the work by Kouassi and Zika (1990). It can also be seen that while the initial rate of change was quite rapid, it slowed down after approximately 10 minutes, stabilising after 25-30 minutes. This therefore suggested that fluorescent emissions should only be measured from parts of the coral that were irradiated for equal periods of time. As it is not practical to excite every part of the coral for 30 minutes, a stepper motor was used which moved the clamping device continuously (as opposed to incrementally) past the u/v beam at a rate of 1.25 mm per minute. Thus, every part of any coral transect was excited for a short, but equal amount of time.

Producing a fluorescent profile: Before a fluorescent profile was produced, the positions of the bright and dull bands were obtained by photographing the coral under u/v light and then measuring the distance between bands. This was done to enable visual observations to be compared with fluorescent profiles. The u/v beam was focused on the tip of the living coral surface by placing a piece of white paper on top of the coral and adjusting the position of the clamping device. The stepper motor was then switched on (thus moving the coral past the u/v beam) and the fluorescence measuring device was left to run in complete darkness. As the PMT output voltage was not linked to an analogue to digital converter, voltages were recorded manually once a minute (i.e. every 1.25 mm). In order to determine whether any error was

introduced as a consequence of machine drift, background noise was recorded before and after the production of a fluorescent profile. However, because background noise was not detected when the amplifier was on the medium sensitivity setting, it was recorded with the amplifier on the high sensitivity setting.

Summary of the technique:

(1) The coral skeleton was excited at either 254 nm or 365 nm. Fluorescent emissions were measured at 450 nm, 500 nm and 550 nm.

(2) In order to maximise the amount of fluorescence recorded by the PMT while minimising the amount of background noise recorded, the following steps were taken: the amplifier was set on the medium sensitivity setting and the diaphragm in front of the PMT was half closed; the operating voltage was set to 800 v; the angle between the lamp and the PMT was set to 60° and the angle between the lamp and the coral was set to 90°.

(3) A u/v cut off filter was used in the emission section of the device to prevent u/v light from the lamp being reflected/scattered off the coral surface and reaching the PMT.

(4) The size of the excitation slit was kept small to enable intra-band measurements to be made.

(5) Because of the effect u/v light has on the fluorescent properties on coral fluorophores, coral skeleton was excited for equal periods of time. This was achieved using a stepper motor which moved the coral in front of the u/v beam at 1.25 mm/min.

6.3.4 Results and discussion: Due to unforeseen technical difficulties in the construction of the device, very little work was done using the 254 nm excitation bandpass filter.

Reproducibility of results: In order to ascertain the errors associated with an individual fluorescent profile, five fluorescent profiles were taken from the same transect. As decreases in fluorescent intensity have been recorded through time when humic acids are excited at 365 nm (see Figure 6.14), the coral was left for 30

minutes before the next run to allow the fluorophores to recover. All transects started from the same position. Results showed that while there was some variation between the transects (the standard deviation as a percentage of the mean was 5.28%, see Appendix A6.4), reproducibility was considered to be high (see Figure 6.15).

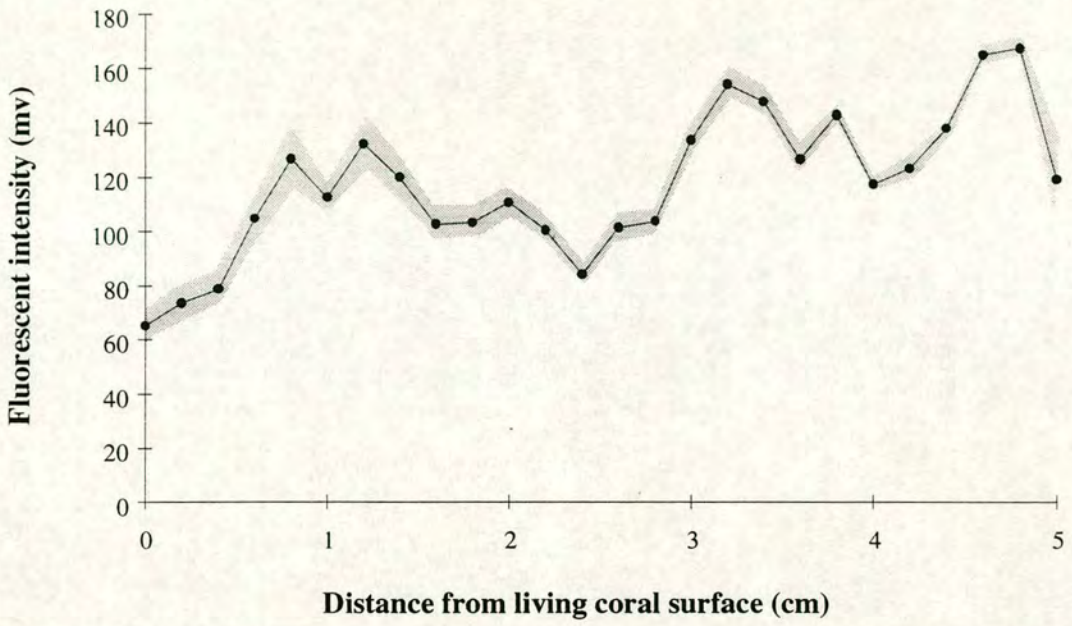


Figure 6.15 shows the variation recorded when five fluorescent profiles were produced from the same coral transect (TS-3-93). The fluorescent profiles were produced by exciting the coral at 365 nm and recording emissions at 550 nm. Solid line represents the mean value while shading indicates the range.

It can be seen in Figure 6.15 that fluorescent intensity increases with increasing distance from the living coral surface. In order to make sure this was not due to some form of machine drift, profiles were measured first one way and then the other along the same transect. Thus, if machine drift has a significant effect, a different profile might be expected for the reverse transect (see Figure 6.16). When this was done for three corals (TS-9-87, TS-5-87, and TS-1-93), very similar fluorescent profiles were produced (see Figure 6.17). The error, calculated as percent deviation away from the mean was 0.99% for TS-9-87, 5.34% for TS-5-87 (although an error of 3.23% was obtained when the reverse transect values were offset by one reading to account for the apparent shift in the data), and 4.10% for TS-1-93.

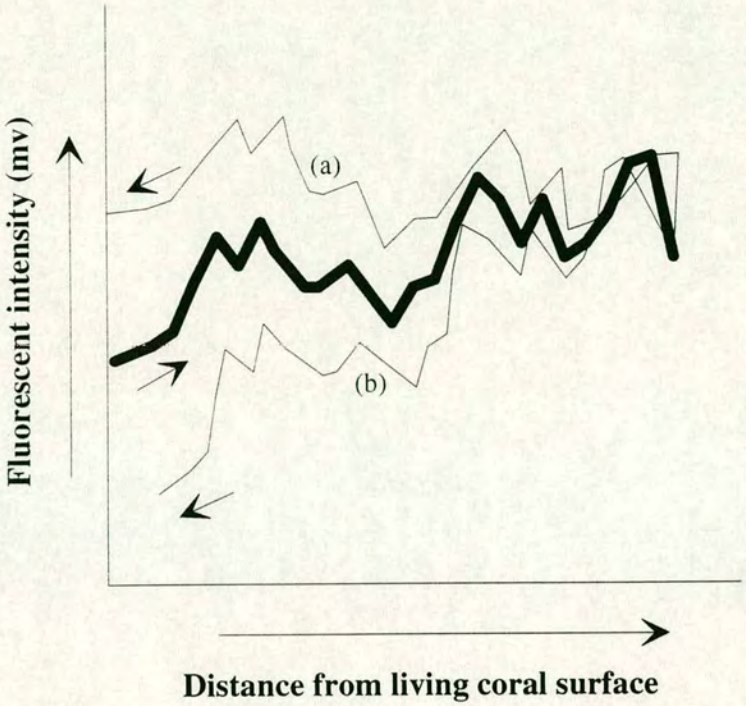


Figure 6.16 The fluorescent transect that might be produced if there is significant instrumental drift to (a) higher and (b) lower values. The bold line represents the forward transect.

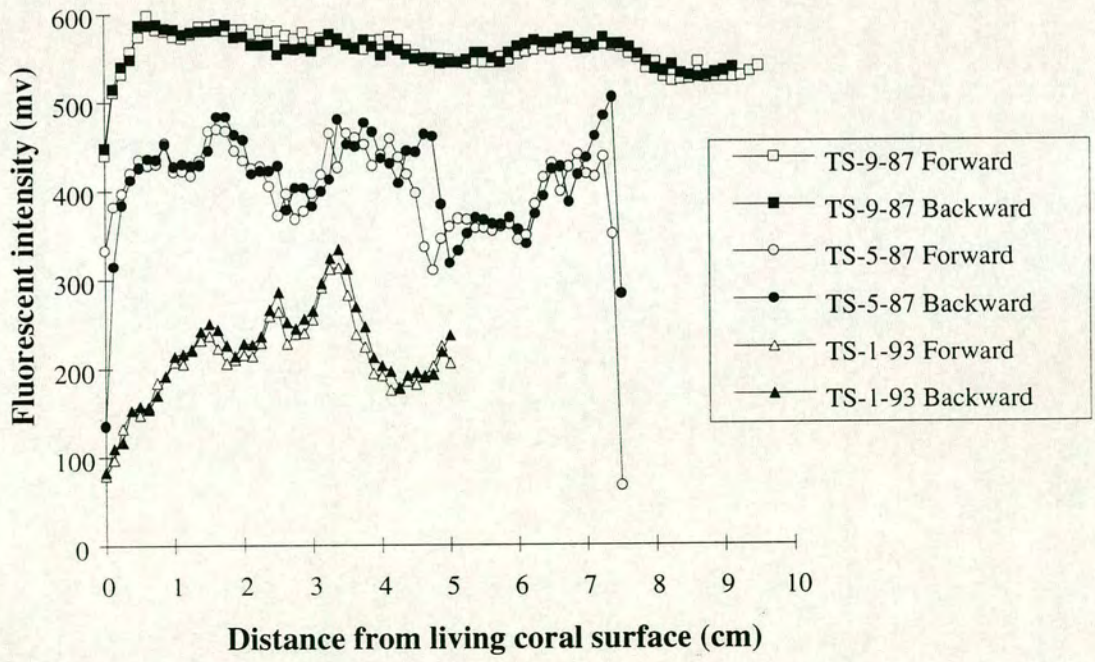


Figure 6.17 The variation produced when transects were taken from opposite ends of the coral. The fluorescent profiles were produced by exciting the coral with 365 nm u/v radiation and recording the emissions at 450 nm.

Thus, these results suggest that the fluorescent profiles produced by the fluorescence measuring device were true representations of the fluorescence emitted by the coral.

The living coral surface: The measurements of fluorescent intensity taken from the living coral surface were always lower than those taken from adjacent parts of the skeleton which were not covered with polyp tissue at the time of collection. Although the reason or reasons for this phenomenon were not discovered, these findings could suggest that either: (1) the u/v beam was not 100% on the coral, or (2) fluorescence was not fully developed in this part of the skeleton. As seen in Figure 6.17, the very first reading taken from the living coral surface was often exceptionally low. This feature was not thought to be due to an initial fluorescent "warm up" period during which fluorescent emissions built up to a maximum, as a similarly low value was also recorded at the other end of the core (see TS-5-87 in Figure 6.17). This edge effect, which probably occurs because the u/v beam is not fully exciting the coral surface, has been used to mark the beginning, and in some cases the end, of fluorescent transects.

Bright and dull band fluorescence: While the fluorescent profiles produced by the fluorescence measuring device were relatively accurate, it was not always easy to determine band type. This was because of the following: (1) fluorescent intensity often increased or decreased away from the living coral surface (see Figure 6.15 on page 190), and (2) the junction between bright and dull bands was not always clearly defined. For these reasons, it was necessary to compare fluorescent profiles with visual observations and measurements of band position to enable their location on the fluorescent profiles to be made. Nevertheless, results showed that the fluorescence measuring device was quite sensitive to small changes in emission intensity and that these changes could be correlated with specific areas. For example, DB3 in TS-5-87 contains a very dark section which corresponds to an area of very low fluorescent intensity. In addition, bright bands are generally more intense than their neighbouring dull bands as shown in Figure 6.18, in which the fluorescent emissions from a single coral were recorded at the three different wavelengths, and in Figures 6.19 and 6.20, in which the fluorescent profiles from a number of corals are shown. These results are therefore consistent with those in Chapter 4 (see Figure 4.9 on page 82) and with those reported by Boto and Isdale (1985), and could reflect higher fluorophore concentrations in bright bands. The fact that TS-3-93 in Figure 6.18 looks different to the profile in Figure 6.15 (on page 190) is because in Figure 6.18, readings were taken every 0.5 cm where as in Figure 6.15 they were taken every 0.2 cm.

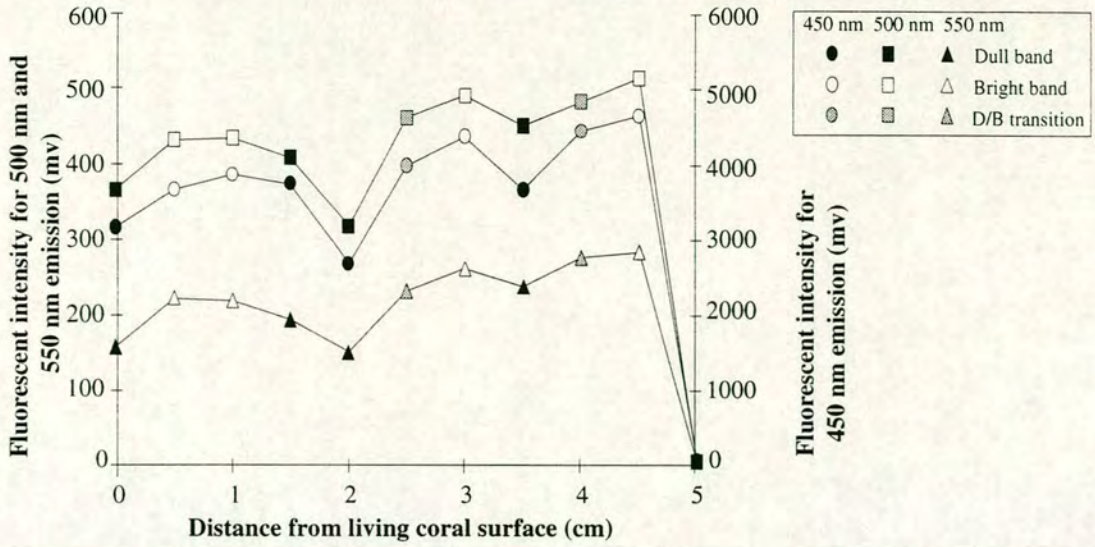
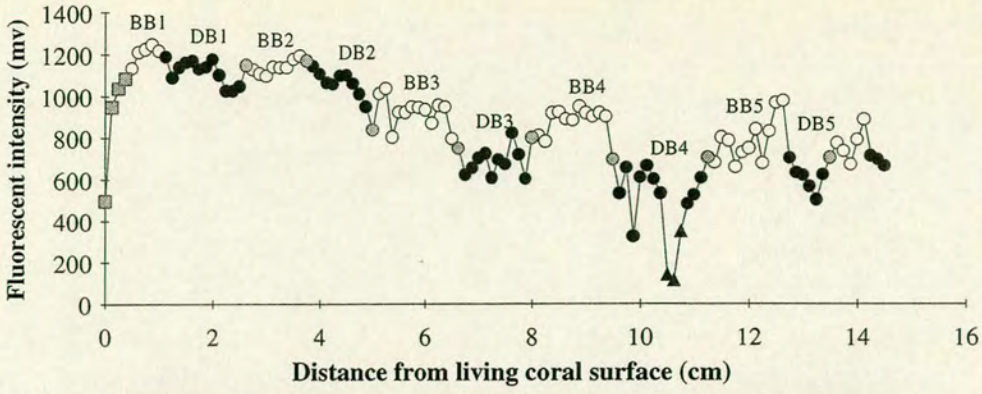
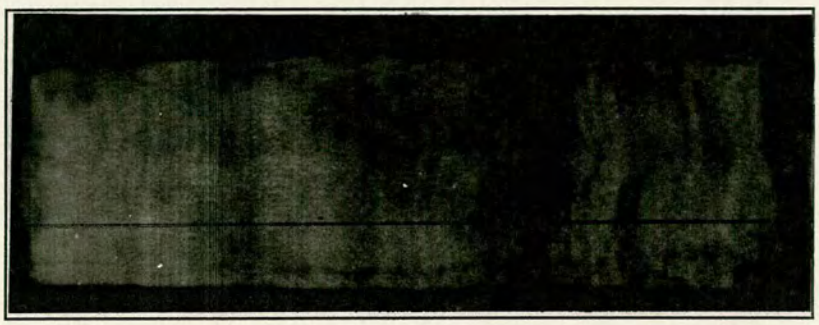


Figure 6.18 Bright and dull band emission intensity at 450 nm, 500 nm and 550 nm when excited at 365 nm for TS-3-93. Data adjusted to PMT variable response. In order to fit all profiles on one graph, the 450 nm emissions have been plotted on a different scale as they were more intense than those at 500 nm and 550 nm. The low value at 5 cm = edge effect.

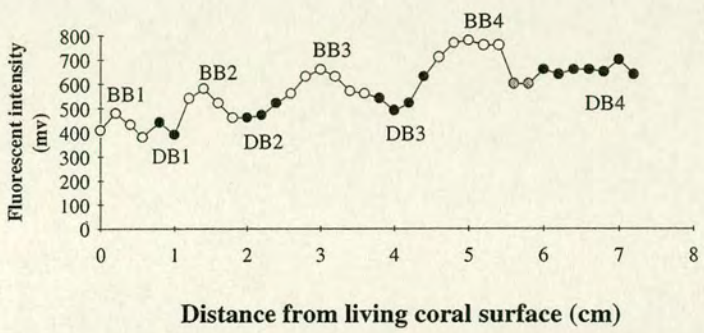
Although bright bands are, in general, more intense than adjacent dull bands, this was not always the case for non-adjacent bands (i.e. some dull bands were more intense than some bright bands). This feature was particularly common when there was a trend towards higher or lower intensities with increasing distance from the living coral surface. This is clearly seen in Figure 6.19 where DB in core 9/1-87 is more intense than BB4 and BB5, and in Figure 6.20 where DB2 in TS-9-87 is more intense than BB4. The fact that very similar fluorescent profiles were produced irrespective of the direction in which the coral skeleton was passed in front of the u/v beam suggested that the trend towards lower (Core 9/1-87, TS-5-93 and TS-9-87) or higher (TS-1-93, TS-3-93 and PB-3-87) emission intensities with increasing distance from the living coral surface was real. Although the reason or reasons for the trends were not absolutely identified, the following explanations are offered. They have been divided into those that are connected with coral preparation and analysis, and those that assume that the trend reflects some environmental parameter.

(a)





(b)



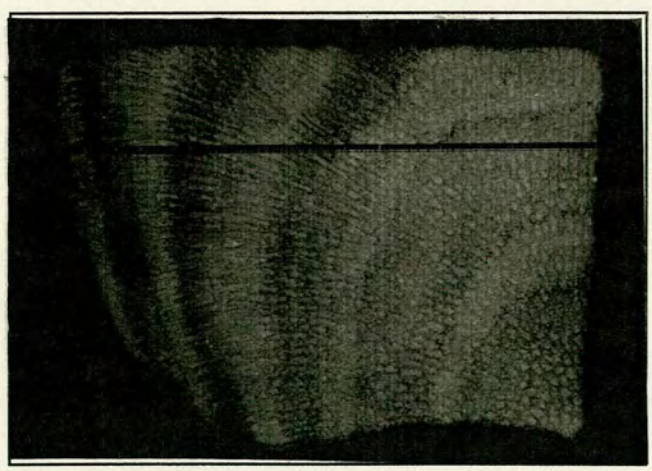
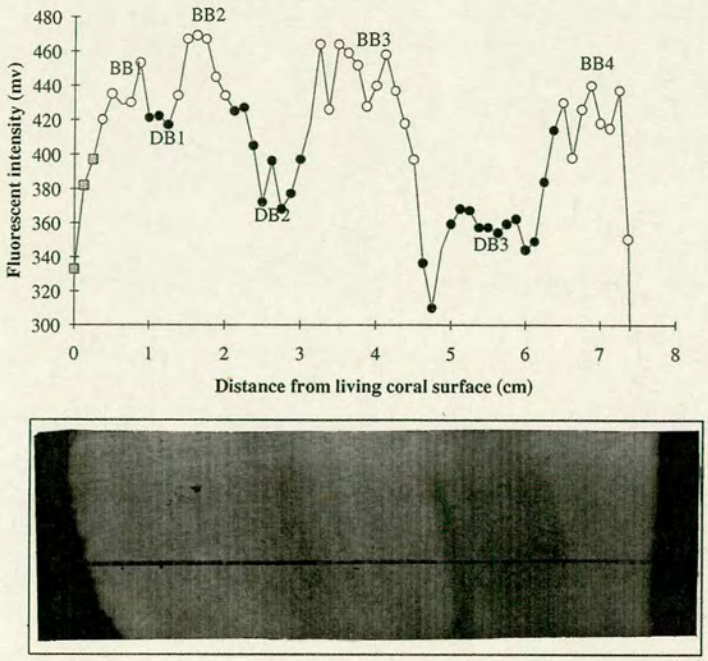
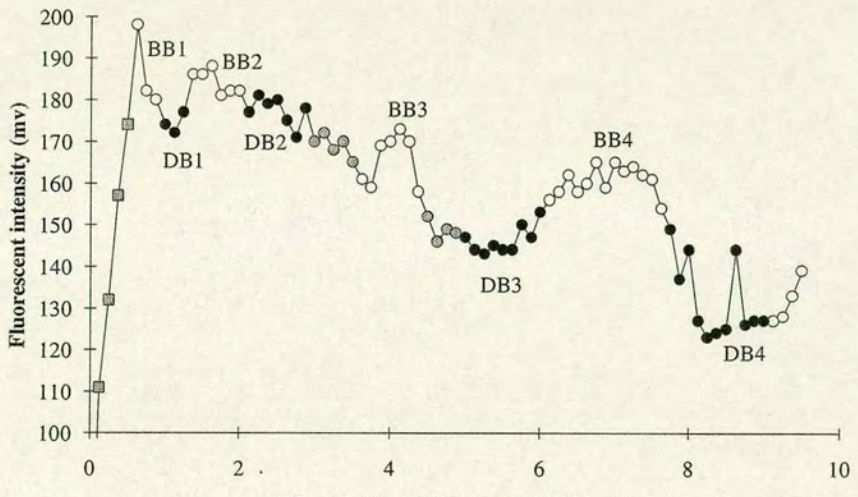


Figure 6.19 Fluorescent profile of (a) Core 9/1-87 and (b) PB-3-87 with associated u/v photographs. Key: Black circles = dull bands; white circles = bright bands; grey circles = bright/dull boundary; grey squares = living coral surface. All photos in Figures 6.18 and 6.19 were taken under even conditions of illumination (i.e. the changes in fluorescent intensity seen are real). The black line marks the position of the transect.

(a)



(b)



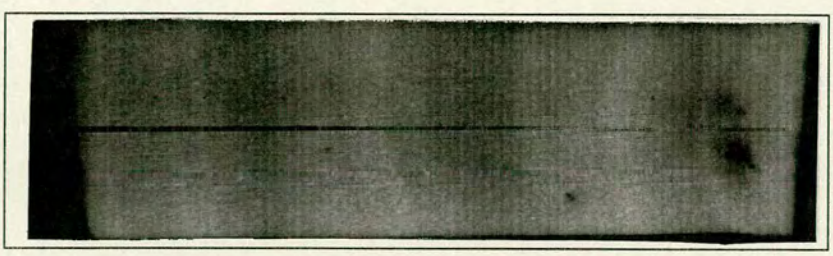


Figure 6.20 Fluorescent profile of (a) TS-5-87 and (b) TS-9-87 with associated u/v photographs. Key: Black circles = dull bands; white circles = bright bands; grey circles = dull/bright boundary; grey squares = living coral surface. The black line marks the position of the transect.

Coral preparation and analysis:

(a) **Corallite orientation**: The orientation of the corallites relative to the u/v beam could be critical as results in Chapter 2, section 2.4, have suggested that surface area plays an important part in the intensity of a fluorescent band. A change from longitudinal to transverse section with increasing distance from the living coral surface was noticed in 9/1/87, TS-5-87, TS-9-87 and PB-3-87. Although this corresponded to a decrease in fluorescent intensity with increasing distance from the living coral surface in 9/1/87, TS-5-87, TS-9-87, it corresponded to an increase in fluorescent intensity with increasing distance from the living coral surface in PB-3-87 (TS-1-93 and TS-3-93 were destroyed before they could be examined). Thus, changes in the orientation of the corallites, which frequently occur in sections longer than 5 cm, do not appear to be the main factor involved in the production of these trends.

(b) **u/v beam focus**: Another factor which could have been involved in the production of these trends involves u/v excitation beam focusing. Although the u/v beam was in focus when the run started, because the machine was left to collect data in complete darkness on its own, it was not possible to determine whether it remained focused all the time.

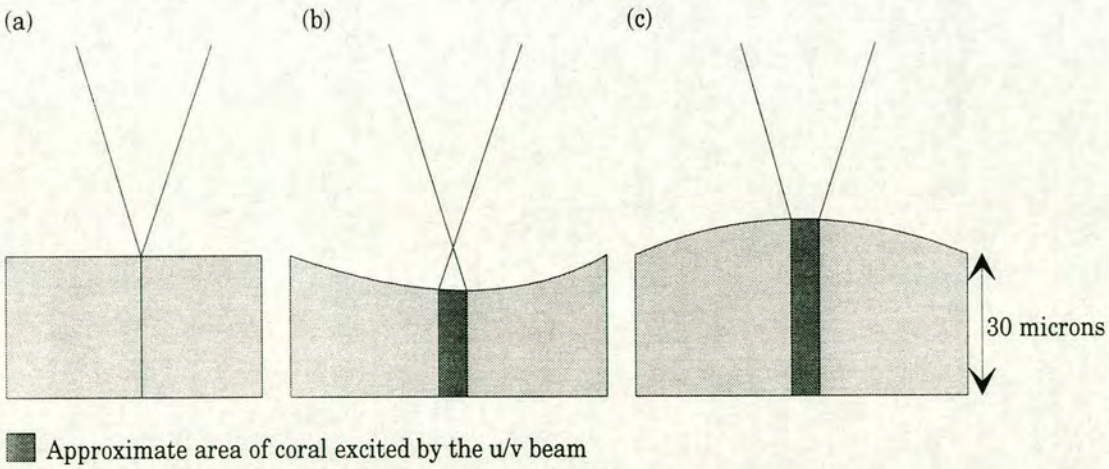


Figure 6.21 The effect that variations in the thickness of the core have on the amount of coral excited by the u/v beam. (a) the beam is focused on the coral surface. (b) when the coral is thinner than it should be or (c) when it is thicker than it should be, the u/v beam is no longer focused and a larger area of coral is excited.

Defocusing may occur because of variations in the thickness of the core (see Figure 6.21). The most likely outcome of such a scenario is that the area excited by u/v radiation would increase which would lead to an increase in fluorescent emissions as more fluorophores would be excited. In other words, if the u/v beam became unfocused at some stage along the transect, one would expect the intensity of fluorescent emissions to increase. However, an increase in fluorescent intensity with increasing distance from the living coral surface was only seen in TS-1-93 (see Figure 6.17 on page 191), TS-3-93 (see Figure 6.15 on page 190 and Figure 6.18 on page 193), and PB-3-87 (Figure 6.19 on page 194), whereas decreases were seen in the other sections. This therefore suggests that beam defocusing was not a significant problem.

Thus, it would seem that coral preparation and analysis is not the main cause of the trends towards increasing or decreasing fluorescent intensity with increasing distance from the living coral surface.

Environmental parameters:

(a) **Ageing of the fluorophores**: Results in Chapter 4 (see Figure 4.3 on page 70) demonstrate that the fluorescent intensity of coral fluorophores in solution decreases over time. Although this is likely to be more rapid in solution than in the solid state, it could, nevertheless, still occur. Evidence to support the hypothesis that some form of fluorophore degradation does occur was provided when old surfaces and freshly cut surfaces from the same coral were examined under a u/v lamp. Results show that the new surfaces are markedly more fluorescent than old ones (see Figure 6.22). It should be noted that these corals were kept in the dark for approximately one year before the fresh surfaces were cut, and as such, the degradation seen should not be due to photo-oxidation.

(b) **The effect of long wave u/v radiation and visible light on coral fluorescence**: Kouassi and Zika (1990) have found that irradiation with visible light results in a permanent decrease in fluorescent intensity. Even though the polyp tissue is likely to offer some protection against fluorophore photo-oxidation, the fact that algal endoliths, which require sunlight for photosynthesis, appear to survive beneath the polyp tissue layer (see Chapter 3, Figure 3.4, page 52), suggests that light can penetrate to at least a depth of 1 cm (the depth below the surface that the living algal endoliths are found). Thus,

fluorophores in the most recently deposited skeleton are more likely to be affected by photo-oxidation in which case, fluorescent intensity may decrease rapidly in the outer few cm's and then stabilise as new skeleton is deposited on top. Certainly, Kouassi and Zika (1990) have suggested that while the breakdown of humic acid by sunlight is initially quite rapid, the breakdown products are much more stable and decompose at a much slower rate thereafter.

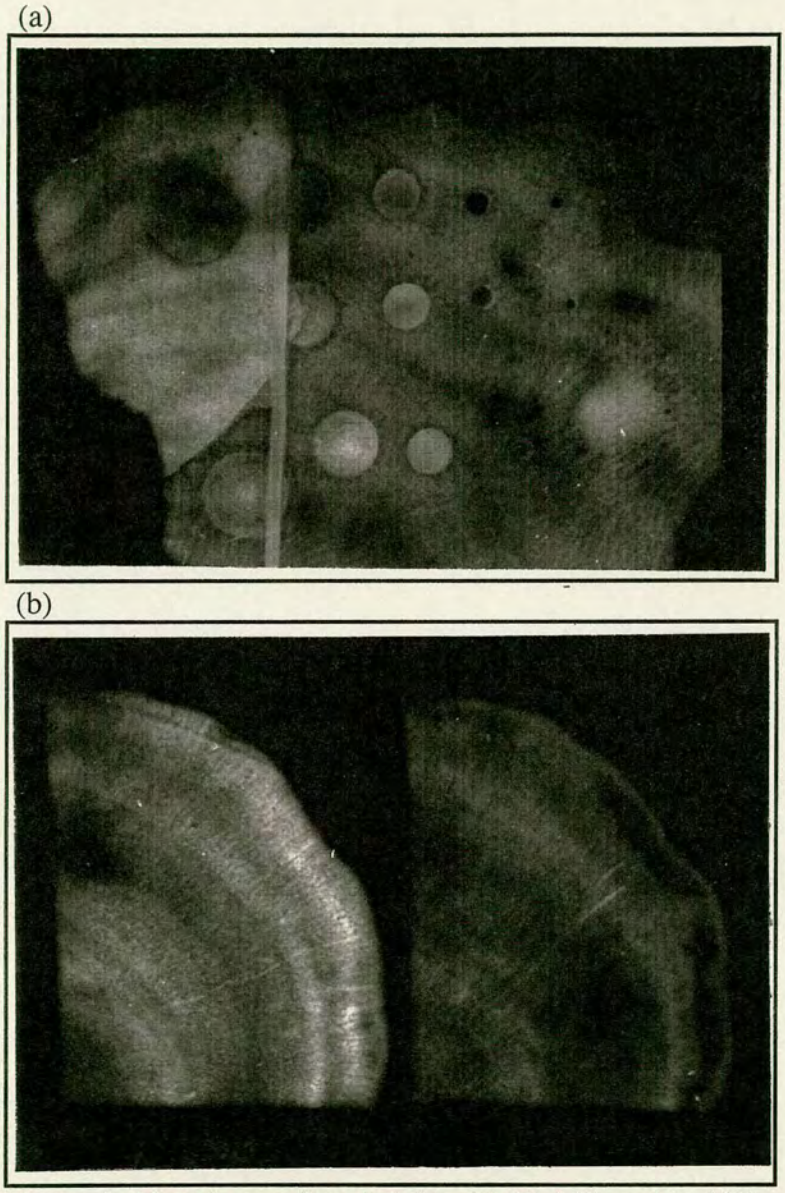


Figure 6.22 New and old surface under u/v light. (a) SB-6-88 used in drilling experiments in Chapter 2 (the sample is 11 cm wide), and (b) PB-2-87 (the sample is 5 cm wide). The freshly cut surface is on the left and the old surface is on the right. Both pieces were dried in an oven for 12 hours to remove water before being photographed. The samples were evenly illuminated so the differences in intensity seen were not due to lighting effects.

(c) **Microbial activity**: Four groups of microscopically active organisms have been implicated in the penetration and destruction of reef structures. These are; sponges, bacteria, fungi and algae. Although they all have the potential to reduce the organic content as a consequence of their boring activities, the effects of fungi and bacteria, which live off organic matter, are likely to be the most significant. Assuming that the microboring organisms do not enhance fluorescent intensity, and results in Chapter 3 suggest that they do not, then as the number of microborings in the skeleton increases, the total organic content of the skeleton is likely to decrease. The most likely outcome of this is that fluorescent intensity will also decrease. Thus, the effect they have on the fluorophore content of coral skeletons is likely to increase with increasing depth as the older parts of the skeleton are likely to have been bored to a greater extent.

Although these models can account for a decrease in fluorescent intensity with increasing distance from the living coral surface, they can not account for the increases seen in PB-3-87, TS-1-93 and TS-3-93. Thus, while all of the above may have some effect on coral fluorescence, they are unlikely to be the main causes of the trends towards increasing or decreasing fluorescent intensity with increasing distance from the living coral surface.

(d) **Fluorophore concentration effects**: In some corals, a reduction in fluorescent intensity is matched by visual observations (9/1-87, PB-3-87, TS-5-87), whilst in other corals it is not (TS-9-87, where BB4 is the brightest looking bright band but was measured to be less intense than BB1, BB2 and BB3). The reason why visual observations do not always match measurements is not obvious. It may be related to changes in colour rather than intensity with the eye being fooled into thinking that a bright band is particularly bright on the basis of a shift to longer emission wavelengths which implies that the energy transfer (ET) threshold has been exceeded. In profiles 9/1-87, PB-3-87, TS-9-87 and TS-5-87, the coral skeleton was excited at 365 nm and fluorescent emissions were recorded at 450 nm. Thus, the relatively low emissions recorded for BB4 in TS-9-87 could be because more fluorescence was emitted at longer wavelengths. In other words, BB4 in TS-9-87 could have a higher fluorophore concentration (above the ET threshold) than either BB1, BB2 or BB3, and as such, emits more light at longer wavelengths. Although this could have been checked by recording the fluorescent emissions at longer wavelengths,

problems with the fluorescence measuring device meant that TS-3-93 was the only coral for which this was done. Results showed that TS-3-93 fluorescence was dominated by short wavelength emissions (i.e. 450>500>550 nm, see Figure 6.18 on page 193). This therefore suggests that the concentration of fluorophores in the coral skeleton as a whole (rather than in the yellow/orange patches) is below the ET threshold. If this is the case, the lower fluorescent intensity of BB4 in TS-9-87 compared to the other bright bands probably reflects a lower fluorophore concentration. Thus, variations in concentration may not be the main cause of the trends towards increasing or decreasing fluorescent intensity with increasing distance from the living coral surface.

As none of the above explanations are entirely satisfactory, it would appear that further work is required in order to establish the cause/causes of the trends towards increasing or decreasing fluorescent intensity with increasing distance from the living coral surface.

6.3.5 Summary/Key points:

(1) Tests demonstrated that the fluorescence measuring device was able to reliably record the intensity of fluorescent emissions from coral skeletons.

(2) Results suggest that fluorescence is dominated by short wavelength emissions (i.e. 450>500>550 nm) which suggests that the concentration of fluorophores in the skeleton as a whole (i.e. blue background region and the yellow/orange fluorescent patches) is below the ET threshold.

(3) Bright bands are, in general, more intense than their neighbouring dull bands, However, trends towards increasing fluorescent intensity with increasing distance from the living coral surface in some profiles and decreasing intensity in others, meant that this was not always the case (i.e. not all bright bands were brighter than every dull band). Although measurements of fluorescent intensity were reliably recorded, it was not possible to determine the reason for these trends. Thus, it was not possible to determine whether the fluorescent profiles produced using the fluorescence measuring device were a true reflection of the concentration of fluorophores present in the skeleton or whether some other factor was involved.

6.4 IS FLUORESCENT BANDING DUE TO DIFFERENCES IN COLOUR, INTENSITY, OR BOTH?:

Before discussing the use of fluorescent profiles for comparison with environmental data, one has to establish whether fluorescent intensity can be used as an environmental indicator. In order to determine this, the question "is fluorescent banding due to differences in colour, intensity or both?" has to be answered.

If there is a colour difference between bright and dull bands in the solid state, the concentration of fluorophores would not only have to be higher in bright bands than in dull bands, but it would also have to be above the ET threshold. This could be achieved if a situation like the one shown in Figure 6.23 existed.

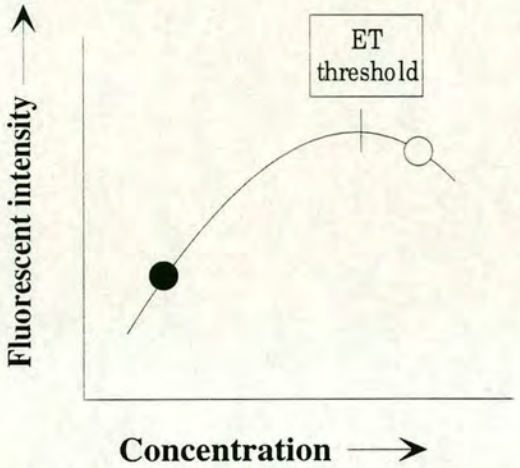


Figure 6.23 Schematic diagram showing how fluorescent intensity of coral fluorophores changes with increasing concentration. Bright band = white circle; dull band = black circle. See Chapter 5, Figure 5.3, page 160 for further details.

However, when solid state fluorescence data (obtained using the LS-5 spectrofluorimeter, see section 6.2, and the fluorescence measuring device, see section 6.3) were examined, short wavelength emissions dominated in both bright and dull bands. This therefore suggested that, irrespective of band type, the concentration of fluorophores in the coral skeleton as a whole was below the ET threshold. As bright band solid state fluorescent intensity is, in general, greater than dull band fluorescent intensity, it would appear that the concentration of fluorophores in bright bands was greater than in dull bands. These results imply that the ET threshold has not been exceeded in which case fluorescent banding is merely an intensity phenomenon. This statement is further supported by Quale (1991), who measured solid state fluorescence from *Porites lutea* and showed that bright bands were up to 100% more intense than dull bands.

While the above strongly suggests that fluorescent banding is an intensity phenomenon, it does not necessarily rule out the possibility that colour differences also exist. The fact that colour predictions failed to detect a difference between solid state bright and dull bands (both blue) when the LS-5 spectrofluorimeter was used (see section 6.2), could be attributed to the PMT's poor response to emissions between 580 nm and 650 nm, and its inability to record emissions above 650 nm[1]. As Boto and Isdale (1985) have presented bright and dull band emission spectra over the whole of the visible spectrum, the colour of fluorescence was calculated using their data to see if a difference could be detected. When this was done, the predicted colour of fluorescence was blue for both bright and dull bands, yet again suggesting that fluorescent banding was due to differences in intensity rather than colour. This could, however, be because their emission data were unadjusted to PMT response. When the PMT correction factor used in Chapter 4 was applied to their data, a difference in the colour of fluorescence was predicted, with bright bands being green and dull bands being blue/green (see Figure 6.24 and Appendix A6.5).

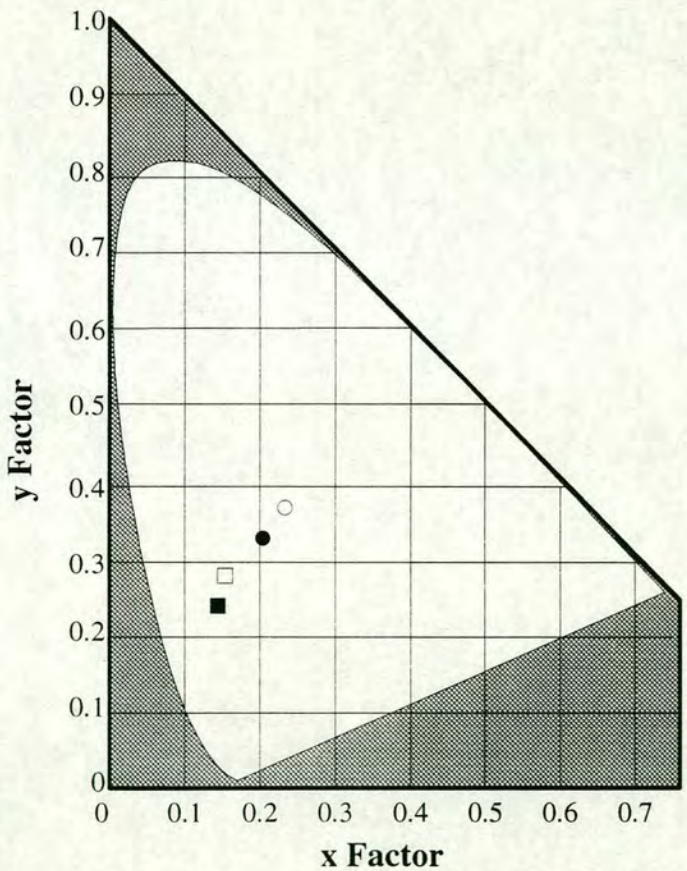


Figure 6.24 The predicted colour of fluorescence for Boto and Isdale's (1985) bright and dull band emission spectra. Key: white = bright band; black = dull band; squares = data unadjusted to PMT response; circles = data adjusted to PMT response. For colours see Chapter 4, Figure 4.14, page 88.

[1]The colour of fluorescence was not calculated using data from the fluorescence measuring device because of a shortage of bandpass filters

Emissions above 580 nm were not included in the calculations for two reasons: (1) a PMT correction factor was not available between 650-700 nm as the PMT could not record emissions over this range, and (2) although a PMT correction factor did exist for emission wavelengths between 580-650 nm, the low sensitivity of the PMT to emissions over this range would have introduced a large degree of error into any colour calculations. Thus, the difference in colour between Boto and Isdale's (1985) bright and dull bands could be even greater than predicted. Perhaps the most convincing evidence in favour of a difference in colour between bright and dull bands is provided by the discovery of yellow/orange fluorescent patches when coral skeleton was viewed using a fluorescence microscope (see Chapter 5, Figure 5.1, page 157).

Thus, the question arises "how can there be a difference in the colour of bright and dull band fluorescence when fluorescent emissions from the skeleton as a whole and irrespective of band type, are dominated by short wavelengths (thus no ET)?"

6.4.1 Producing a difference in colour: For there to be a difference in colour between the two band types, the concentration of fluorophores in at least one of the bands (i.e. the bright bands) has to be above the ET threshold. Although results have shown that this is certainly not the case in the skeleton as a whole, the concentration of fluorophores in the yellow/orange patches is thought to be above the ET threshold, hence the yellow/orange fluorescence. Thus, a situation like the one shown in Figure 6.23 does exist in the skeleton, but only in small patches. Without these concentrated fluorophore patches, it is unlikely that colours with wavelengths longer than blue would be seen. This is because the concentration of fluorophores in the rest of the skeleton (i.e. the background region) would appear to be too low for significant energy transfer to occur (hence the blue fluorescence).

Thus, a colour difference can exist between the bands. The actual colour seen will, however, ultimately depend on the ratio of background fluorescence to yellow/orange fluorescence (not just the number of yellow/orange patches to background fluorescence but also the relative intensities of each). In other words, the colour seen will ultimately depend on how much yellow/orange light is mixed with the blue background fluorescence. However, what is emitted is not necessarily what is seen by the eye (see Chapter 4, Figure 4.17, page 94), as the eye does not respond to all wavelengths equally. The fact that bright bands are not yellow/orange when excited by longwave u/v light is probably because the vast majority of the skeleton

emits a blue background fluorescence. However, because our eyes are not as good at seeing blue light as they are at seeing yellow/orange light (see Chapter 4, Figure 4.12, page 86), the yellow/orange fluorescent patches have a larger effect on the colour seen than would otherwise have been expected. Thus, even though results in this chapter and chapters 4 and 5 have demonstrated that coral fluorescence, irrespective of band type, is dominated by short wavelength emissions (i.e. between 400 nm and 500 nm, purple to blue[2]), there is enough fluorescence emitted at longer wavelengths to result in a slight shift in the colour of fluorescence seen towards the green/yellow (which is why neither of the bands are purple even though this might be the colour expected from EXEM spectra). The fact that the background fluorescence to yellow/orange fluorescence ratio would appear to be higher in bright bands (i.e. bright bands have more yellow/orange patches than dull bands), could result in a greater shift towards the green/yellow than in dull bands and hence a difference in colour.

Although a difference in colour between bright and dull band coral solutions is possible, it is much less likely for the following reasons: (1) in the solution phase, the fluorophores were assumed to be homogeneously distributed as a consequence of diffusion, and as a result, localised areas of high fluorophore concentrations are unlikely to exist, (2) the fluorophores are no longer isolated from the other molecules in the same way as they were in the solid state and can therefore interact with each other, and (3) there is a considerable dilution factor involved when making coral solutions (between 10 and 30). The above ensure that the concentration of fluorophores in the solution, irrespective of band type, is below the ET threshold. As shown in Chapter 4 (see section 4.6.1, pages 97-114), when fluorophore concentration is below the ET threshold, differences in concentration are manifest as differences in intensity which is probably why most 1M coral solutions had a blue fluorescence irrespective of band type.

6.4.2 Producing a difference in intensity: The fact that fluorescent emissions from the skeleton as a whole are dominated by short wavelength emissions (i.e. 400 nm to 500 nm) is probably because the vast majority of the skeleton emits a blue fluorescence. In others words, fluorescent emissions from the background region of the skeleton dominate yellow/orange patch fluorescence. This, therefore, suggests that the concentration of fluorophores, in what probably amounts to more than 95%

[2]This is also the case for the yellow/orange fluorescent patches.

of the skeleton, is below the ET threshold. Whether this means that the intensity of coral fluorescence as a whole is controlled solely by the concentration of fluorophores in the background region of the skeleton, with bright band background having a higher concentration of fluorophores than dull band background, was not absolutely determined. The picture is somewhat complicated by the presence of the yellow/orange fluorescent patches which appeared, on visual inspection, to be more fluorescent (i.e. more intense) than the blue background fluorescence (see Chapter 5, Figure 5.1, page 157). It is conceivable that these yellow/orange fluorescent patches add enough intensity to the background fluorescent signal to control fluorescent intensity (i.e. bright bands are more intense because they have more yellow/orange patches than dull bands). However, if this were the case, then a larger amount of longer wavelength fluorescence than is at present recorded would be expected. Perhaps the most likely scenario is that both the yellow/orange patches and the background fluorescence affect the intensity of coral fluorescence. As it was not possible to calculate the relative intensities of either the background fluorescence or the yellow/orange fluorescence, it is not possible to say which of the two sources of fluorescence would play a greater part in controlling the final fluorescent intensity recorded. However, the fact that short wavelength emissions are so dominant would suggest that the background region of the coral is the main control on coral fluorescent intensity. As the concentration of fluorophores in the background region of the skeleton is below the ET threshold, fluorescent intensity can be used as an environmental indicator.

6.4.3 The concentration of fluorophores in the skeleton: One final point relates to the estimates of fluorophore concentration in coral skeletons. A 144 ppm Fluka humic acid solution, with absorbing impurities, was required to produce a green/yellow fluorescence when excited at 360 nm, while the same concentration solution without the absorbing impurities only produced a green colour when excited at 360 nm (see Chapter 4, Figure 4.27, page 108). Thus, it is reasonable to assume that the concentration of humic acid required to produce yellow/orange fluorescent patches would be higher than 144 ppm, although exactly how much higher is not known at present. As the concentration of fluorophores in the blue background region of the skeleton would appear to be very low, and as the yellow/orange fluorescent patches are so few in number, the concentration of fluorophores in the yellow/orange patches would have to be very high indeed if the mean fluorophore concentration for the whole of the skeleton was to be between 140 ppm and 420

ppm. As it was not possible to determine the concentration of fluorophores in a typical yellow/orange fluorescent patch, this question remains unanswered.

6.4.4 Summary/Key points:

(1) The fact that areas of very high fluorophore concentration (i.e. above the ET threshold) can exist in the coral skeleton is probably the main reason why colours other than blue can be emitted, even though the concentration of fluorophores in either band as a whole is only high enough to produce blue fluorescence. The final colour of fluorescence emitted is primarily controlled by the ratio of yellow/orange fluorescence to background fluorescence. As the concentration of the former increases, the colour of fluorescent emissions shifts to longer wavelengths.

(2) The fact that the vast majority of the coral skeleton emits blue fluorescence suggests that the concentration of fluorophores in the background region of the skeleton is below the ET threshold. Although the effect of the yellow/orange patches on the total fluorescent intensity recorded was not determined, the dominance of emissions between 400 nm and 500 nm suggests that its effects are relatively small compared to background fluorescence. Work in Chapter 4 has demonstrated that below the ET threshold, changes in the concentration of fluorophores results in changes in fluorescent intensity. The observation that bright bands are in general more intense than dull bands could, therefore, suggest that the concentration of fluorophores in the background region of bright bands is greater than in the background region of dull bands. As the intensity of coral fluorescence would appear to be controlled by part of the coral in which fluorophore concentration is below the ET threshold, fluorescent intensity can be used as an environmental indicator.

(3) The fact that only indirect evidence exists to support the hypothesis that bright bands are both brighter and lighter than dull bands was attributed to: (1) the inability of the LS-5 to accurately record emissions over a wide range of wavelengths, and (2) a shortage of bandpass filters for the fluorescence measuring device.

6.5 CAN FLUORESCENT PROFILES BE CORRELATED WITH ENVIRONMENTAL PARAMETERS?:

Although the reason for the trends towards increasing or decreasing fluorescent intensity with increasing distance from the living coral surface were not absolutely identified, a correction factor can be applied which removes them. By doing this, it may be possible to correlate the fluorescent profiles generated by the fluorescence measuring device with rainfall, sunshine or productivity.

6.5.1 Data manipulation: Converting fluorescent profiles into monthly records of fluorescent intensity is a rather subjective process. Although stain experiments have clearly shown that the dull/bright band transition occurs around November, and that the bright/dull band transition probably occurs between July and August, the effect that polyp tissue has on coral fluorescence at the living coral surface has made it difficult to determine where the first genuine dull/bright band transition occurs. The position of the most recent dull/bright band transition was assigned on the basis of fluorescent couplet thickness (see Figure 6.25). In Figure 6.25a, the November 1986 dull to bright band transition in TS-5-89 could also have been put at (F). As the fluorescent couplets AC, BD and CE were 3.2 cm, 2.5 cm and 2.3 cm respectively, if (F) represented the November 1986 dull to bright band transition, it would not be unreasonable to assume that the coral should grow by an approximately similar amount by November 1987. The fact that (F) is 0.5 cm from the living coral surface (which in this case is August 1987) would mean that in the nine months since November 1986, it had only grown 0.5 cm. Thus, in the remaining three months, it would have to grow at least 1.5 cm. Therefore, as it seems unlikely that the couplet will more than double in size over the next three months even if linear extension rate in dull bands is greater than in bright bands (see Chapter 7, Table 7.1, page 230), (F) is unlikely to represent the November 1986 dull to bright band transition. In a similar way, the position of the November 1986 dull to bright band transition was assigned in the other corals.

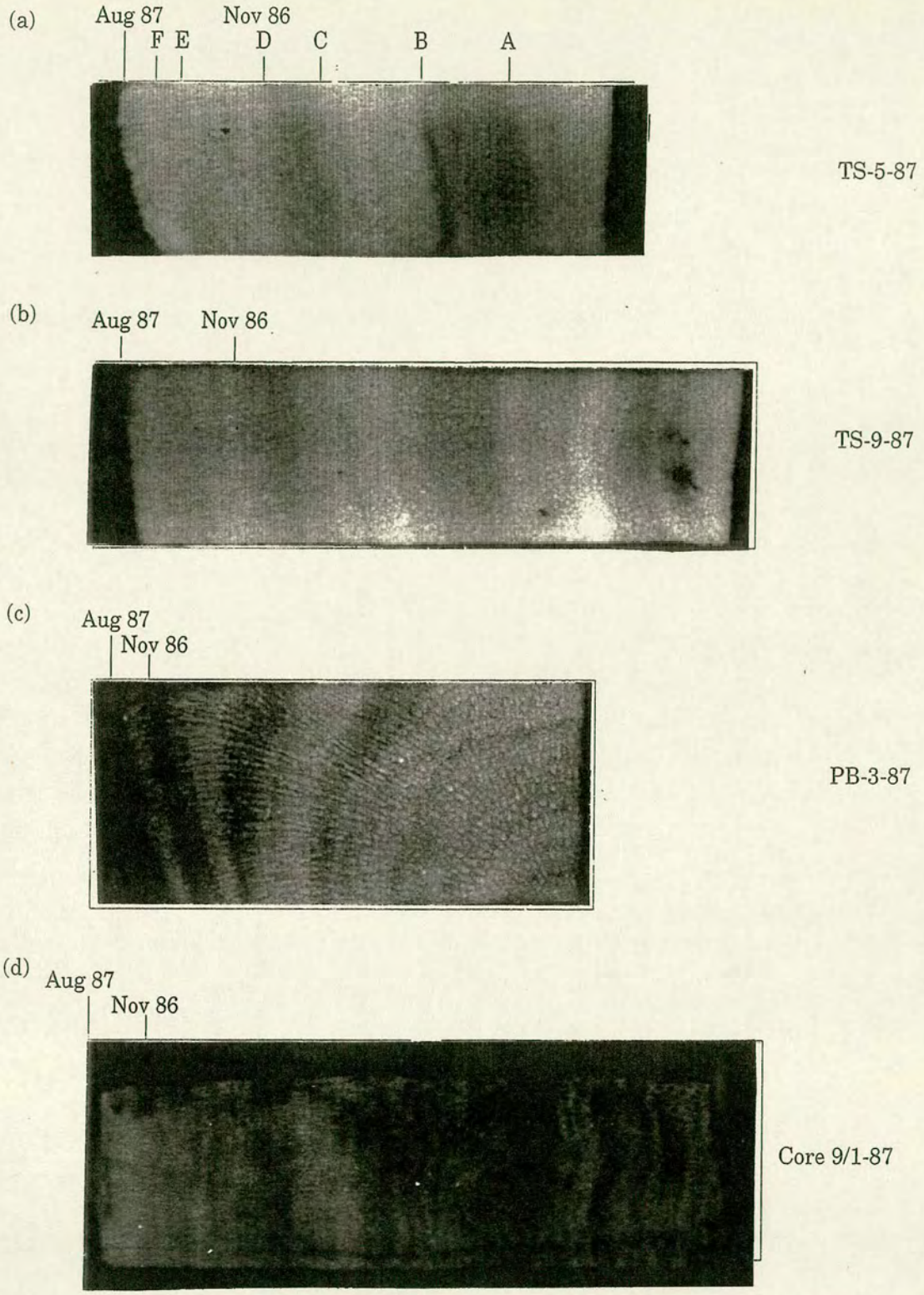


Figure 6.25 The position of the November 1986 dull to bright band transition. (a) TS-5-87, (b) TS-9-87, (c) PB-3-87, (d) core 9/1-87.

As linear extension rates will vary from year to year and perfect longitudinal sections are difficult to obtain, the number of measurements taken from individual fluorescent couplets varied. For example, the fluorescent couplet AC in TS-5-89 will contain more measurements of fluorescent intensity that the fluorescent couplet BD because the former is longer. As environmental data was provided in monthly form (i.e. 12 values/year), a direct comparison was, therefore, not possible. Thus, the fluorescence data had to be converted into monthly form before it could be compared with the environmental data. This was achieved using an equal spacing program which took the fluorescent data obtained using the fluorescence measuring device, and calculated what the fluorescence would be at various intervals (in this case months), assuming a fluorescent couplet is deposited every twelve months. When fluorescence was plotted against rainfall, it appeared that periods of high rainfall coincided with dull band deposition (see Figure 6.26).

However, by fixing the date of the dull/bright transition to November and assuming that a fluorescent couplet represents a year, then a reasonably good correlation with rainfall, which also shows a seasonal signal in this area, would be expected (i.e. the correlation produced merely tells you that the two signals have the same sort of seasonality). Even though a correlation coefficient close to 1 may be obtained when this is done, it does not necessarily mean that the two series are causally related. Thus, it was considered necessary to remove the seasonality from the fluorescent (and ultimately environmental) records in order to see whether periods of abnormally high fluorescence could be linked with similar peaks or troughs in environmental records. In order to achieve this, mean and standard deviation fluorescent values were calculated for each month. Each individual monthly figure was then expressed as an anomaly from the monthly mean in standard deviation units. In order to remove the trends towards increasing or decreasing fluorescent intensity with increasing distance from the living coral surface, an exponential curve was fitted to the seasonally adjusted data. This curve was then assumed to represent a zero value and the variation away from zero (in standard deviation units) was then calculated for each value. Both forms of data (i.e. raw data and exponentially adjusted data) are presented in this section.

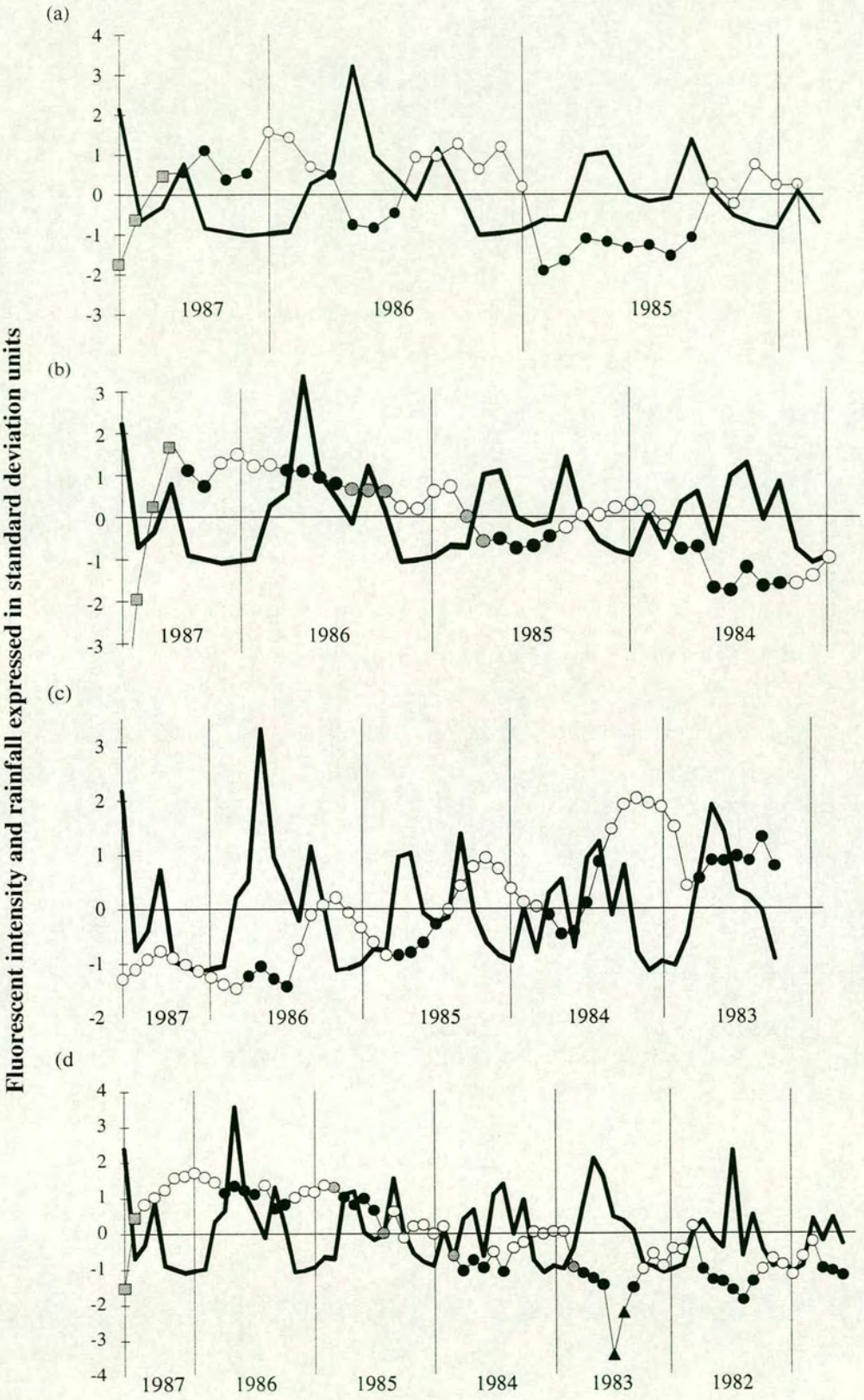


Figure 6.26 Fluorescence versus rainfall. (a) TS-5-87, (b) TS-9-87, (c) PB-3-87 and (d) core 9/1-87. Symbols: white circles = bright bands; black circles = dull bands; grey circles = dull/bright band boundary; grey squares = living coral surface; black triangles = iron band.

6.5.2 Can coral fluorescence be used as an environmental indicator? If coral fluorescence is to be used as an environmental indicator, it is important to know how consistent fluorescent banding is within a colony, between colonies on the same reef, and between colonies on different reefs. In order to determine whether fluorescent banding was consistent within an individual colony, three fluorescent profiles were produced from TS-3-93 (see Figure 6.27). Although the fluorescent profiles were 1 cm apart, the same fluorescent bands were covered.

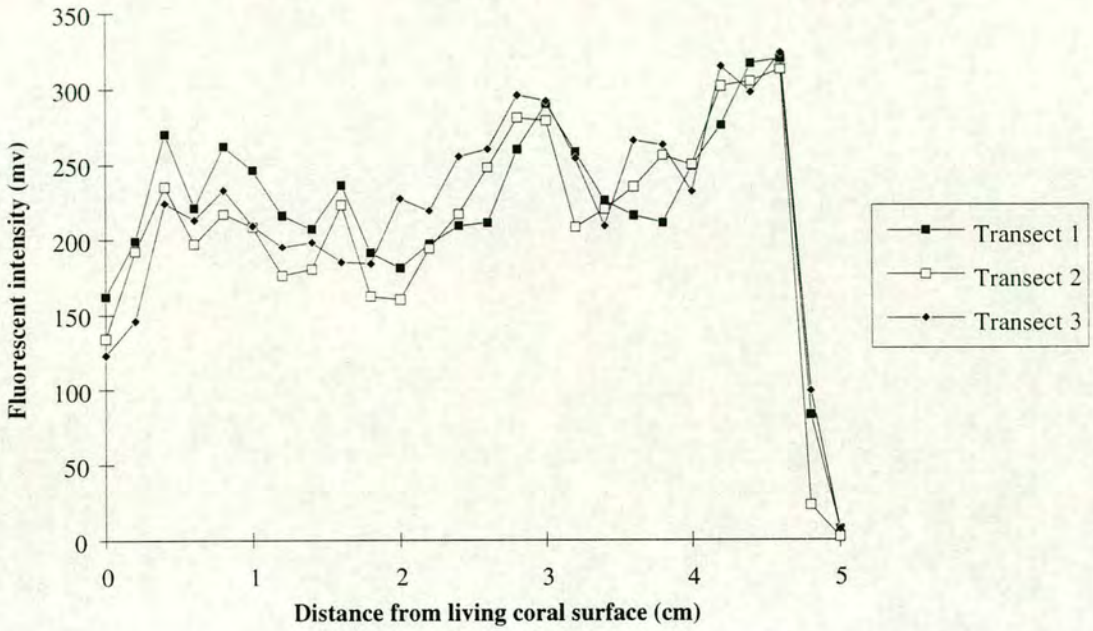


Figure 6.27 Variations in fluorescent intensity from three transects taken over the same bands from the same coral (TS-3-93).

When Pearsons correlation coefficient (r) was calculated for the data in which the seasonality had been removed (see Figure 6.28a), a value of 0.80 was obtained. When a Students 't' test was performed, a value of 6.94 was obtained which was significant at the 99% confidence level (n=30). However, part of this good correlation is due to the fact that fluorescent intensity increases with increasing distance from the living coral surface (i.e. it reflects the fact that the profiles are sloping). When the trends were removed (see Figure 6.28b), an (r) value of 0.44 was obtained. When a Students 't' test was performed, a value of 2.56 was obtained which was significant at the 95% confidence level (n=30).

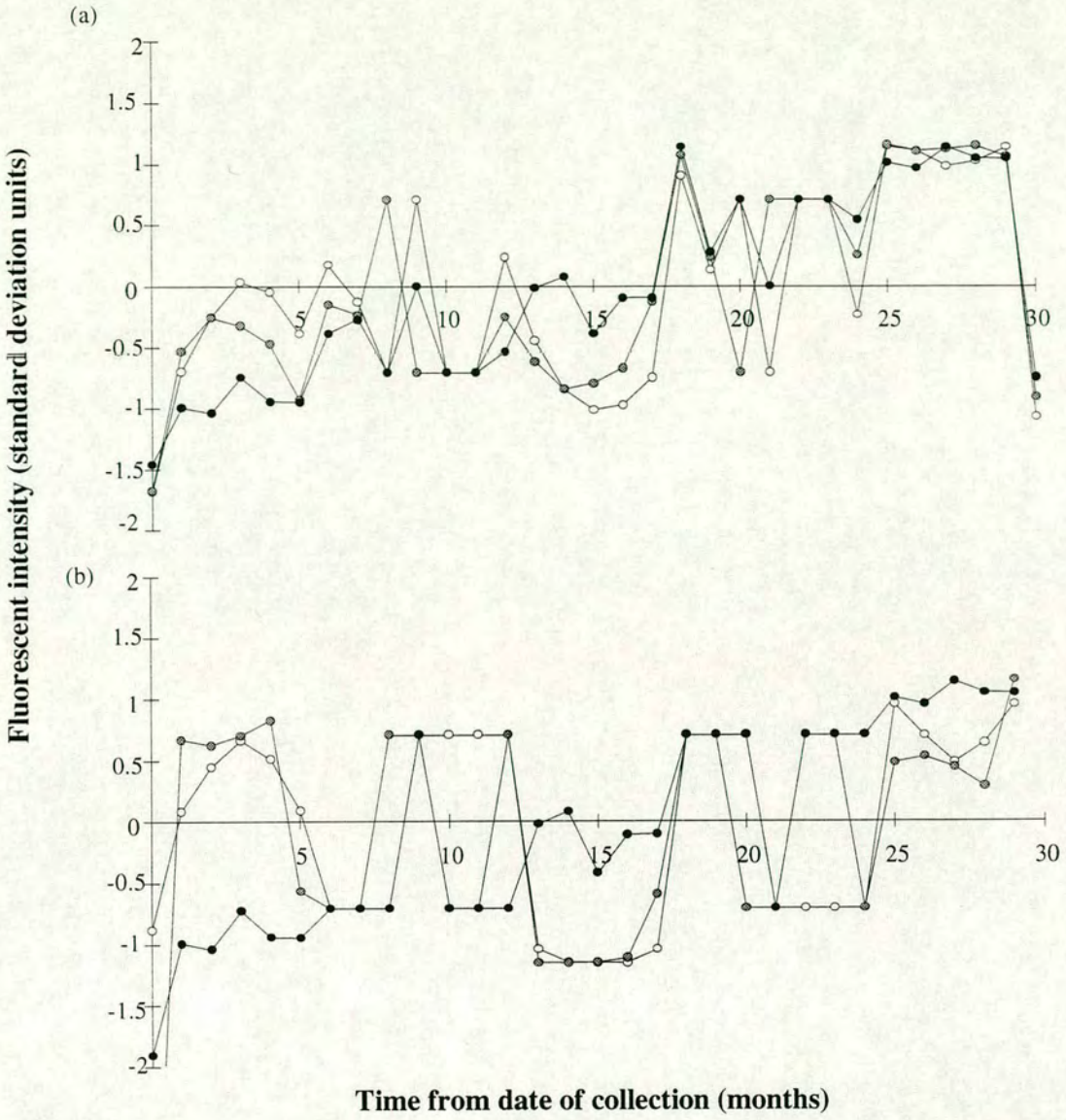


Figure 6.28 Variations in fluorescent intensity from three transects taken over the same bands from the same coral (TS-3-93). The transects were taken 1 cm apart. Data has been adjusted to remove (a) seasonality, (b) seasonality and trends.

These results therefore suggested that fluorescent banding patterns were moderately consistent over short distances. Table 6.1 presents the (r) values that were calculated when fluorescent profiles from different colonies (some from different reefs) were compared.

Table 6.1 Correlation coefficients for coral versus coral.

Samples	Raw	Exp adj
TS-9-87 with TS-5-87		
(r)	0.56	0.14
(r^2)	0.31	0.02
t-test	**3.68**	0.76
9/1-87 with TS-9-87		
(r)	0.94	-0.36
(r^2)	0.88	0.13
t-test	**15.19**	**2.16**
TS-9-87 with PB-3-87		
(r)	-0.26	0.26
(r^2)	0.07	0.07
t-test	1.46	1.50
9/1-87 with PB-3-87		
(r)	-0.06	-0.38
(r^2)	0.00	0.14
t-test	0.35	**2.26**
9/1-87 with TS-5-87		
(r)	0.59	-0.09
(r^2)	0.34	0.01
t-test	**3.96**	0.51
TS-5-87 with PB-3-87		
(r)	-0.08	0.08
(r^2)	0.01	0.01
t-test	0.47	0.42

Key: Raw = raw data not adjusted to an exponential curve; Exp adj = data adjusted to an exponential curve. It should be noted that the values presented in this table were calculated using fluorescence data from the two corals that covered the same period of time. In other words, when the seasonality was removed and standard deviation units calculated, the same number of months were included for both even though TS-9-87 was a longer record. The critical t-test value is 2.04 at the 95% confidence interval. Values which a significant relationship was found have been highlighted.

The high (r) values for the correlations between 9/1-87 and TS-9-87, and between 9/1/-8 and TS-5-87 using the raw data are probably due to the fact that in these profiles, fluorescent intensity decreased with increasing distance from the living coral surface. This is also probably the reason why low (r) values were obtained when these corals were correlated with PB-3-87 in which fluorescent intensity increased with increasing distance from the living coral surface. This, therefore, demonstrates the need to remove the trends. However, given the limitations of the fluorescence measuring device and the small data sets, fluorescent profiles were not consistent for corals collected from the same or different reefs, even when the data had been adjusted to account for sloping trends. Although such low correlations may have been expected for corals on different sides of the peninsula as environmental conditions could be quite different, the values obtained between Porites Bay and Tin

Smelter Bay corals are unlikely to be due to different conditions as the two reefs share part of the same bay (see Chapter 4, Figure 4.45, page 142). The poor correlations seen in Table 6.1 and the reason why (r) values closer to 1 were not obtained for the three transects from TS-3-93 could be due to the following:

(a) Although work in Chapter 2 (see section 2.4, pages 32-43) has suggested that the number and orientation of pore spaces can affect fluorescent intensity, the extent of the effect has not been determined.

(b) The heterogeneous distribution of fluorophores within the skeleton could result in a range of fluorescent intensity readings for adjacent parts of a particular band. The effects of this are, however, likely to be reduced by the u/v beam which is 2 mm wide (i.e. the beam will have some soothing effect).

(c) Correlations, either between two coral fluorescent profiles or between a coral fluorescent profile and an environmental parameter, will depend on the exact position of the band transitions. This is because peaks/troughs in fluorescence have to be assigned a point in time to be correlated with other fluorescent profiles or environmental parameters. The position of a transition will therefore determine the position of peaks/troughs in fluorescence within a certain year. As the transition from one band to another is often gradual, the precise position of the boundary between bands was difficult to determine, both visually and using fluorescent profiles (see section 6.3). This could therefore introduce unwanted variability when comparing fluorescent profiles.

6.5.3 Summary/Key points: At present, the very poor correlations produced, even when data collected from the same reef were compared, prevents any useful correlation with environmental parameters being done. Thus, it was not possible to determine whether fluorescent banding in *Porites lutea* from Ko Phuket is related in some way to rainfall or some other environmental parameter. More reliable data could be produced by:

(a) Generating a master curve for each colony. This would involve taking several transects from one core and making a master curve for that core. Combine the master curves from a number of cores to make a master curve for the colony. By doing this, the variation caused by: (1) the heterogeneous distribution of

fluorophores, (2) pore spaces, and (3) the 3D nature of fluorescent banding (i.e. LS to TS in 3 cm), should be reduced.

(b) Impregnating the cores with black epoxy resin. However, this would: (1) permanently disfigure the coral, and (2) make fluorescent profiles (which are based on intensity) density related (i.e. the more pore space excited, the lower the intensity of fluorescence recorded). This could be avoided if a very fine u/v beam could be produced (i.e. 10 μm to 20 μm) and a number of spot readings from exposed were averaged from each band.

6.6 CONCLUSIONS:

(A) The much greater fluorescent intensities recorded for solid coral over coral solutions is thought to be due to the following: (1) very high fluorophore concentrations in the solid state, and (2) greater absorption of incident radiation in the solid state. Although a difference was seen between bright and dull bands in the solid state when viewed under a u/v lamp, no such difference was detected in their EXEM spectra. This was attributed to the poor response of the PMT to emissions over 580 nm. Thus, a PMT that could record accurately over the whole of the visible spectrum may be able to detect a difference between bright and dull bands.

(B) Because reproducibility was poor and only small samples (less than 10 cm long) could be analysed, the LS-5 spectrofluorimeter was not extensively used to measure solid state fluorescence. A fluorescence measuring device was therefore developed which satisfied both these criteria. When fluorescent banding was clearly discernible to the naked eye, the fluorescence measuring device was able to distinguish between adjacent bright and dull bands, with bright bands being more intense than adjacent dull bands. However, when fluorescent banding was less well defined, it was necessary to compare fluorescent profiles with visual observations to allocate peaks and troughs in the fluorescent profile with specific fluorescent bands. In addition to this, fluorescent intensity was seen to increase in some fluorescent profiles and decrease in others with increasing distance from the living coral surface. As it was not possible to determine whether these trends were genuine or an artefact of the technique, the quality of the data is questionable. Even when the slopes were removed, the quality of the data was not sufficiently high to warrant further statistical analysis at this stage.

(C) Coral fluorescence in the solid state is probably both a colour and an intensity phenomenon which arises as a consequence of the heterogeneous distribution of fluorophores within the skeleton. Coral fluorescent intensity is probably controlled by the combined effects of yellow/orange fluorescence and background fluorescence. However, the fact that short wavelength emissions dominate suggests that background fluorescence is the main control on fluorescent intensity. As the concentration of fluorophores in the background region of the skeleton would appear to be below the ET threshold for both bright and dull bands, the intensity of the fluorescence emitted is a good indication of the concentration of fluorophores in the skeleton as a whole. As bright bands are in general more intense than dull bands, this tends to suggest that the concentration of fluorophores in bright bands is higher than in dull bands. The primary control on the colour of fluorescence is thought to be the ratio of yellow/orange fluorescent patches to background fluorescence. As the concentration of the former increases, the colour of fluorescent emissions shifts to longer wavelengths. However, due to our eyes' uneven response to colour, the yellow/orange patches have a much larger effect than might otherwise be expected which means that only a small number need be present to have a large effect.

(D) The fact that yellow/orange patches exist as isolated patches in the skeleton is probably the reason why they can produce yellow/orange fluorescence there. When solutions were made, the fluorophores in these patches probably become evenly distributed throughout the solution and as such, their effective concentration is greatly reduced. Thus, much higher fluorophore concentrations would be needed in the solutions to produce bright band fluorescence.

CHAPTER 7. WHY ARE BRIGHT BANDS DEPOSITED IN THE DRY SEASON IN *PORITES LUTEA* FROM KO PHUKET?

CHAPTER 7. WHY ARE BRIGHT BANDS DEPOSITED IN THE DRY SEASON IN *PORITES LUTEA* FROM KO PHUKET?

7.1 INTRODUCTION:

Work in Chapter 6 demonstrated that it was not possible to correlate the fluorescent profiles generated by the fluorescence measuring device with environmental parameters such as rainfall, sunshine and productivity, as the cause or causes of the trends towards increasing or decreasing fluorescent intensity with increasing distance from the living coral surface were not absolutely determined. Thus, it is not possible to confirm or refute the statement made by several authors that rainfall and fluorescent banding are positively related (Isdale 1984; Boto and Isdale 1985; Smith *et al* 1989; Klein *et al* 1990). The aim of this chapter is, therefore, to discuss possible mechanisms that could account for the fact that bright bands are deposited in the dry season in *Porites lutea* from Ko Phuket while dull bands are deposited in the wet season. Explanations have been divided into those that assume that seasonal variations in the supply of terrestrial humic acids into the marine environment are in some way responsible for bright band deposition, and those that assume that fluorescent banding can occur without the need for seasonal variations in the supply of terrestrial humic acids.

7.2 SEASONAL VARIATIONS IN THE SUPPLY OF TERRESTRIAL FLUOROPHORES IN TO THE MARINE ENVIRONMENT: THE KEY TO FLUORESCENT BANDING:

This section assumes that it is seasonal variations in the supply of terrestrial fluorophores into the marine environment that controls fluorescent banding and therefore the timing of bright band deposition. It also assumes that some factor or factors is/are responsible for bright band deposition during the dry season in Thailand even though seawater fluorophore concentrations are higher during the wet season in this area.

7.2.1 Time lag: As experiments involving the use of alizarin red stain have indicated that dull bands are deposited during the wet season (see Chapter 1, Figure 1.5, page 10), if periods of high rainfall are associated with bright band deposition as

suggested by Boto and Isdale (1985), then some form of time lag must exist. Three possible mechanisms are proposed.

The rate at which seawater fluorophore concentration increases in response to the wet season: Although the wet season in Thailand starts in April and ends in November, the onset of each season is not marked by an abrupt increase or decrease in rainfall. Thus, it is reasonable to assume that the supply of terrestrial fluorophores into the marine environment will not suddenly increase the moment the wet season starts or suddenly decrease the moment it ends. As a consequence of this, the concentration of fluorophores in the nearshore marine environment is more likely to increase gradually as the wet season begins and decrease gradually when it finishes. It could conceivably take up to several months of increased rainfall before fluorophore concentrations reached levels that were high enough for bright bands to be deposited, thus introducing a time delay between the onset of the wet season and bright band deposition. In addition to this, if bright bands are to be deposited during the dry season as stain experiments suggest, the terrestrial fluorophores released into the marine environment during the wet season would not only have to remain in the nearshore environment for quite some time after the wet season had ended, they would also have to be relatively resistant to chemical/physical decay. As this project was based in the UK, I was unable to test this hypothesis. However, the action of tides, ocean currents, u/v and visible light make this a rather unlikely scenario, especially as seawater fluorophore concentration appears to be higher during the wet season.

The possibility that coral fluorophores reside in the polyp tissue: Work by Davies (1993) has shown that TiO_2 can reside in the polyp tissues for up to three months. If something similar were also to happen to coral fluorophores, their incorporation into the coral skeleton would be delayed. However, work by Susic *et al* (1991) has shown that periods of increased seawater and coral skeleton humic acid concentration occurred at the same time in samples collected from the Great Barrier Reef. This would therefore suggest that the uptake of fluorophores into the skeleton occurs fairly rapidly which implies very little residence time in the polyp tissue.

A time delay due to the manner in which $CaCO_3$ is deposited: A third possible way of producing a time lag was suggested from work done by Barnes and Lough (1993) on density banding in *Porites spp.* They suggested that over the course of several months, the amount of $CaCO_3$ deposited as a consequence of thickening (i.e.

$CaCO_3$ deposition not at the coral surface) within the polyp tissue layer was approximately equal to that deposited at the coral surface. Assuming a linear extension rate of 11 mm/year and a polyp tissue layer thickness of 5.4 mm, they calculated that any part of the coral skeleton could be overlain by polyp tissue for up to six months. The manner in which they believe $CaCO_3$ is deposited is shown schematically in Figure 7.1.

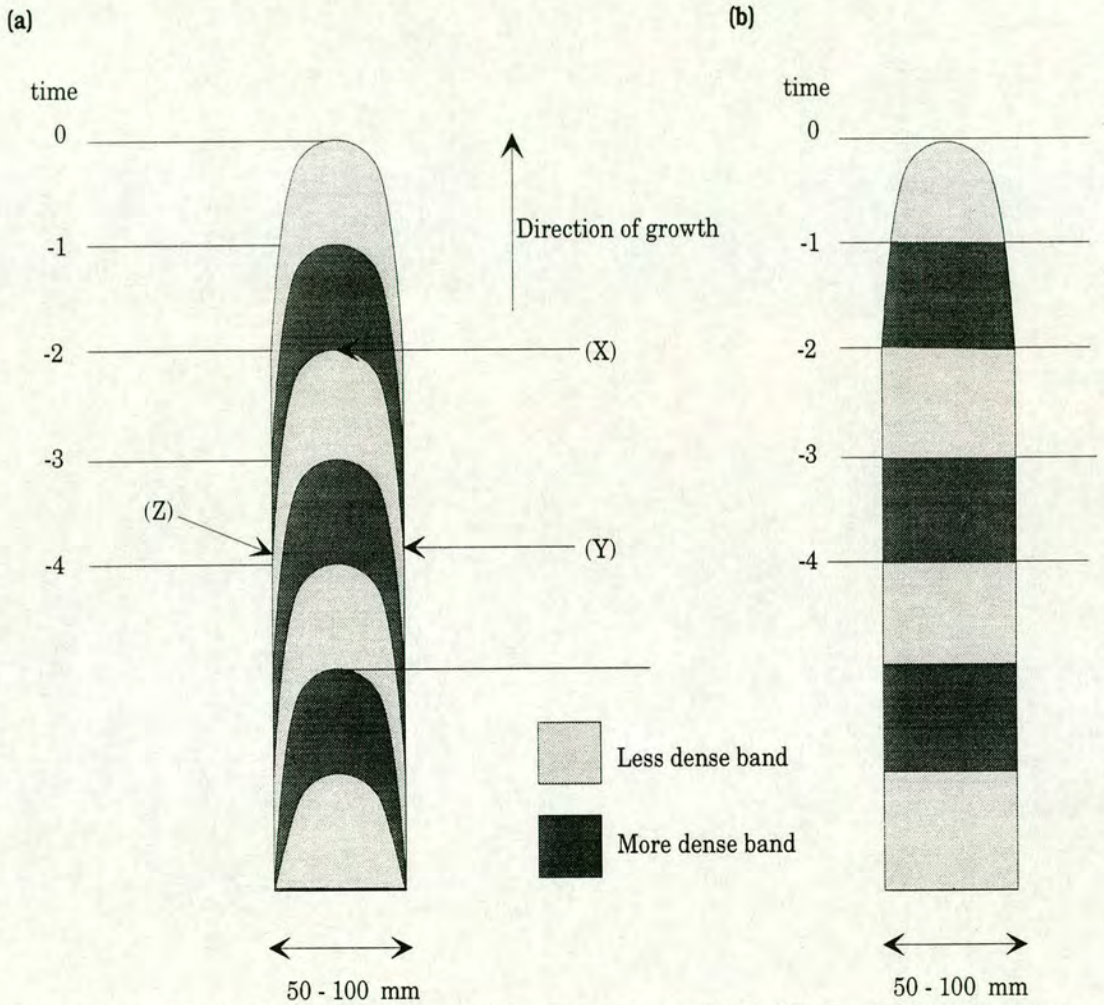


Figure 7.1 Schematic diagram showing (a) Barnes and Lough's (1993) proposed mechanism for $CaCO_3$ deposition, and (b) the more conventional model for $CaCO_3$ deposition. The time units are arbitrary and LCS stands for living coral surface.

Figure 7.1 is a simplified cross-sectional representation of part of a corallite wall in which the direction of linear extension has been indicated. In order to illustrate how Barnes and Lough's (1993) model for $CaCO_3$ deposition could affect fluorescent banding, let us consider what happens to an imaginary point (X) as the coral grows. It can be seen that at point (X), which represents the living coral surface at time (-2), less dense material was being deposited. As the coral grew, point (X) was overlaid

first by dense material and then by less dense material so that at the present day, time (0), the less dense material deposited at time (-2) actually appears at points (Y) and (Z). This gives the impression that the less dense material deposited at time (-2) formed at time (-4) i.e. earlier than it actually did. Barnes and Lough (1993) suggested that this offset could be anywhere between one and eight months. If $CaCO_3$ really is deposited in this way, then a similar 1-8 month offset would be expected for fluorescent bands. However, there are a number of weaknesses associated with this model:

(a) One might expect fluorescence across a corallite wall to be banded. However, when the coral skeleton was examined using a fluorescence microscope, no evidence of fluorescent layering could be detected. This does not necessarily condemn their hypothesis, as work in Chapter 5 (see Figure 5.1, pages 157-158) has shown that fluorophore distribution is heterogeneous, even on a very small scale (i.e. within a corallite wall). Thus, even though $CaCO_3$ may be deposited in layers, fluorescence does not necessarily have to look layered.

(b) According to Barnes and Lough (1993), the corallite wall should be thinner below a dissepiment[1]. This is because the abrupt upward movement of the polyp during dissepiment formation would mean that the coral skeleton immediately below the dissepiment would not have been thickened for as long as other parts of the skeleton. Although numerous thin sections were examined at magnifications between x100 and x600, observations suggested that thinning beneath the dissepiments was by no means a general phenomenon.

To summarise, it would appear that a six month time lag, either as a consequence of the supply and up take of terrestrial fluorophores or because of the way in which $CaCO_3$ is deposited, is unlikely to be the main reason why bright bands are deposited in the dry season.

7.2.2 The hydrodynamics in Phangnga Bay: As airflow from the south west causes net surface currents to flow towards the north of Phangnga Bay during the wet season (SW Monsoon) (Siripong *et al* 1987), fluorophore-rich water could be transported away from the field area towards the head of the bay. This water would be replaced by fluorophore-depleted fully marine water which could, therefore, result

[1]Dissepiments in *Porites lutea* are manifest as horizontal plates which form between the corallite walls.

in the deposition of dull bands during the wet season. During the NE Monsoon (dry season), airflow from the NE causes net surface currents flow towards the south of Phangnga Bay (Siripong *et al* 1987). Thus, it is possible that locally generated fluorophores could mix with fluorophores from north of study area (i.e. from the mangrove swamps at the head of Phangnga Bay) thereby increasing the concentration of fluorophores in the waters around Ko Phuket at this time thus resulting in the deposition of bright bands during the dry season. As corals are not found at the head of Phangnga Bay (Tudhope *pers. comm.*), it was not possible to test this hypothesis. However, if the timing of fluorescent band deposition is controlled by the hydrodynamics of Phangnga Bay, one might expect the concentration of fluorophores in the seawater around the reefs in the study area to be lower during the wet season and higher during the dry season. Results in Chapter 4 (see Figure 4.44 on page 140) have shown that the concentration of fluorophores in seawater is higher during the wet season than the dry, while work by Kouassi and Zika (1990) questions the ability of fluorophores to travel large distances without being photo-oxidised. Thus, it would appear that the hydrodynamics of Phangnga Bay are probably not the main cause of bright band deposition during the dry season.

7.2.3 Fluorescence and sunshine: Kouassi and Zika (1990) have reported that u/v and visible light affect the fluorescent properties of humic acids (also see Chapter 6, Figure 6.14, page 187). They suggested that while the effects of u/v light are largely reversible, the effects of visible light are permanent and destructive. They also suggested that the different behaviour of humic acids to long and short wavelength u/v light is due to the presence of more than one type of fluorophore. Work in Chapter 4 has identified possibly three different types of fluorophores in coral skeletons which could all behave differently when exposed to u/v and visible light. In order to test the effects of u/v and visible light on coral fluorescence, three 20 mls aliquots of a 5M coral solution were prepared. As discussed in Chapter 4 (see section 4.2.2, page 67) the molarity of a particular coral solution refers to the calcium molarity of that solution. One solution was exposed to daylight for seven days, a second was left under a u/v lamp for seven days and the third was kept in the dark for seven days as a control. Before analysis on the LS-5 spectrofluorimeter, each sample was made up to 20 mls with distilled water (DH_2O) (replacing water lost as a consequence of evaporation) and adjusted to pH 9. It should be noted that the solutions were not kept in a vacuum during the experiment and therefore some oxidative degradation was possible; this was not, however, a major cause for concern as the effects of oxidative degradation should be approximately the same for each

solution. An excitation/emission (EXEM) spectrum of the control solution was not taken at the beginning of the experiment because results in Chapter 4 (see Figure 4.5 on page 77) have shown that because of the long-term variation associated with the LS-5 spectrofluorimeter, the fluorescent intensities of solutions collected on different days should not be compared. Bearing these factors in mind, results show that the action of u/v and visible light reduces the fluorescent intensity of both test solutions relative to the control, especially the one exposed to u/v light (see Figure 7.2).

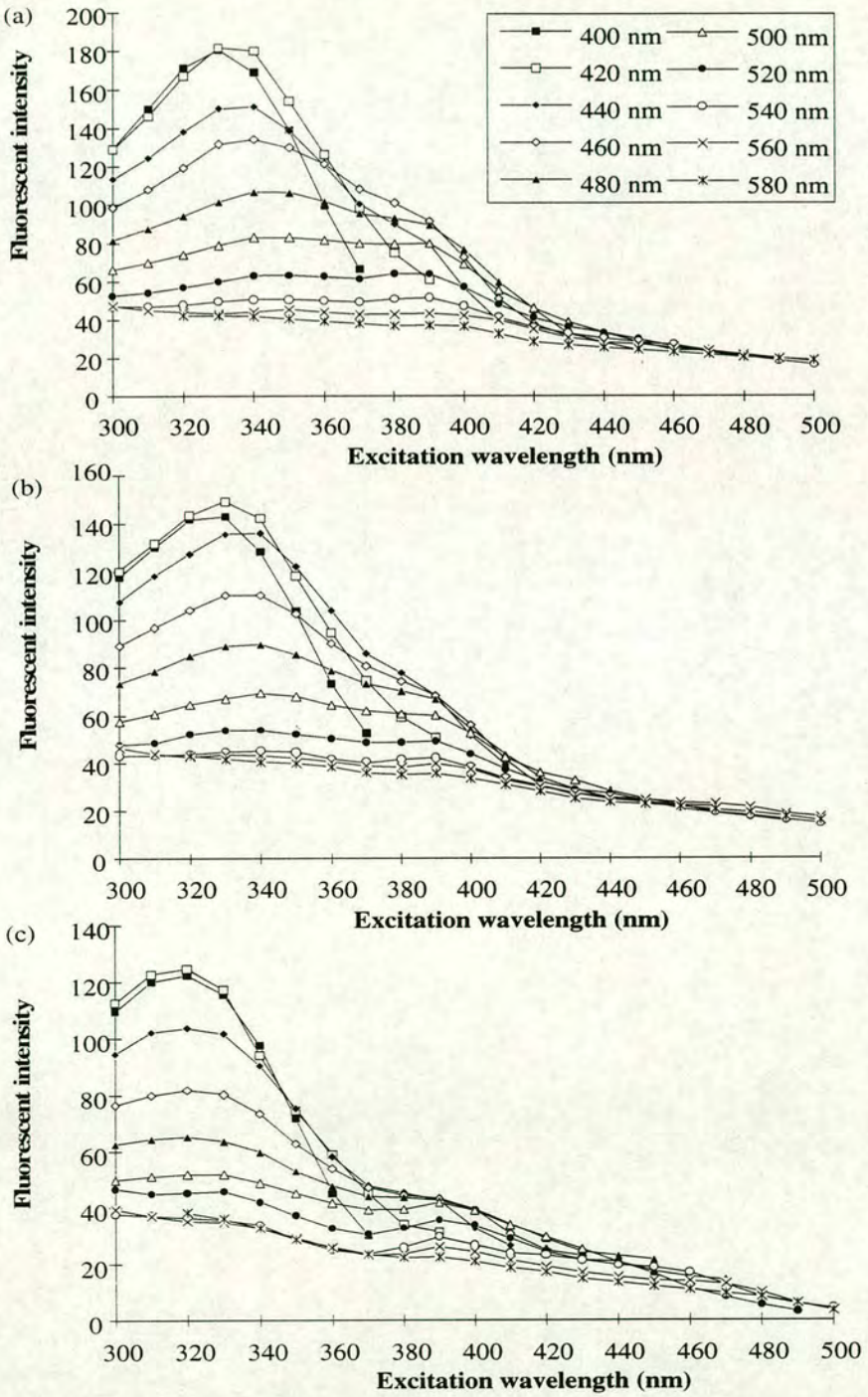


Figure 7.2 The effect u/v and visible light have on coral fluorescence. (a) control solution, (b) exposed to visible light for seven days, (c) exposed to u/v light for seven days.

It did not, however, result in a significant change in the predicted colour of fluorescence when the solutions were excited at 330 nm, 360 nm and 390 nm (see Figure 7.3). Similar results were obtained when three 20 ml aliquots of a 72 ppm Fluka humic acid solution were treated in the same way (also shown in Figure 7.3).

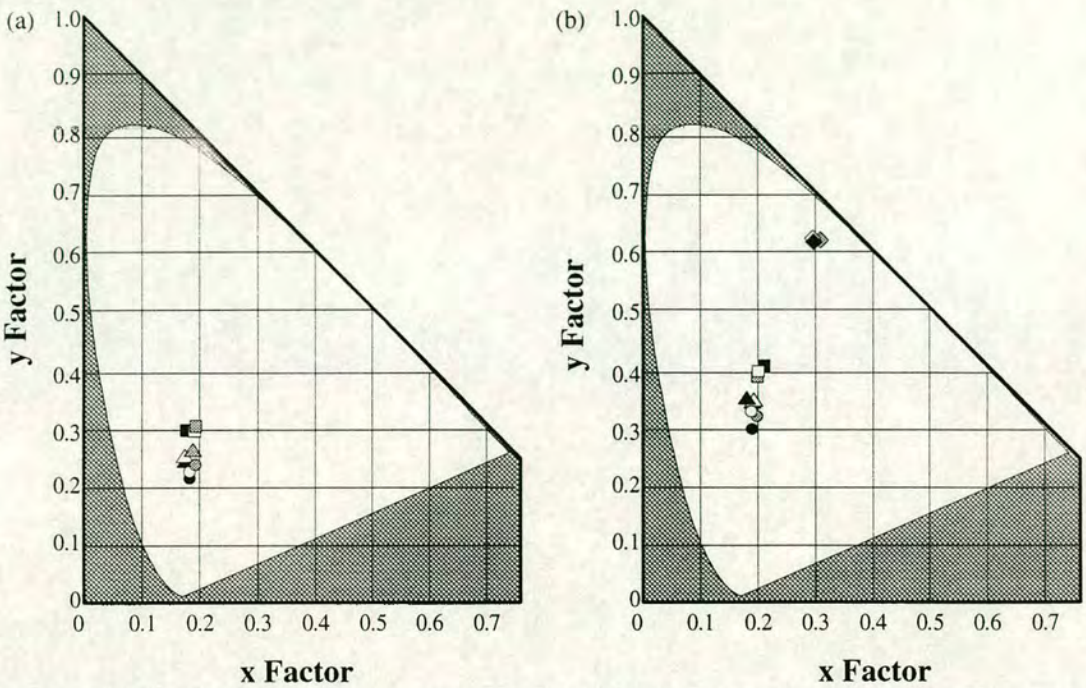


Figure 7.3 The effect that exposure to u/v and visible light for seven days has on the fluorescent properties of (a) a 5M coral solution and (b) a 72 ppm Fluka humic acid solution. Key: circles = excitation at 330 nm; triangles = excitation at 360 nm; squares = excitation at 390 nm; diamonds = excitation at 470 nm (for Fluka humic acid only); white = exposure to visible light for seven days; grey = exposure to u/v light for seven days; black = kept in the dark for seven days (control). Refer to Chapter 4, Figure 4.14 on page 88 to assign colours to these points.

Although the decrease in fluorescent intensity may have been rapid, as suggested by Kouassi and Zika (1990), the action of u/v and visible light did not result in the complete destruction of coral or humic acid fluorescence. Kouassi and Zika (1990) suggest that this could reflect the formation of stable fluorophore complexes which are much slower to break down. Therefore, it is conceivable that coral fluorophores could travel large distances and still be reasonably fluorescent. Therefore, the decrease in the size and possibly fluorescent intensity of bright bands with increasing distance from the mainland could reflect the effects of u/v and visible light on coral fluorophores.

However, rather than resulting in the deposition of bright bands during the dry season when sunshine hours are at a maximum (see Figure 7.4), the effects of

increasing exposure of coral fluorophores to u/v and visible light are more likely to result in the deposition of dull bands at this time.

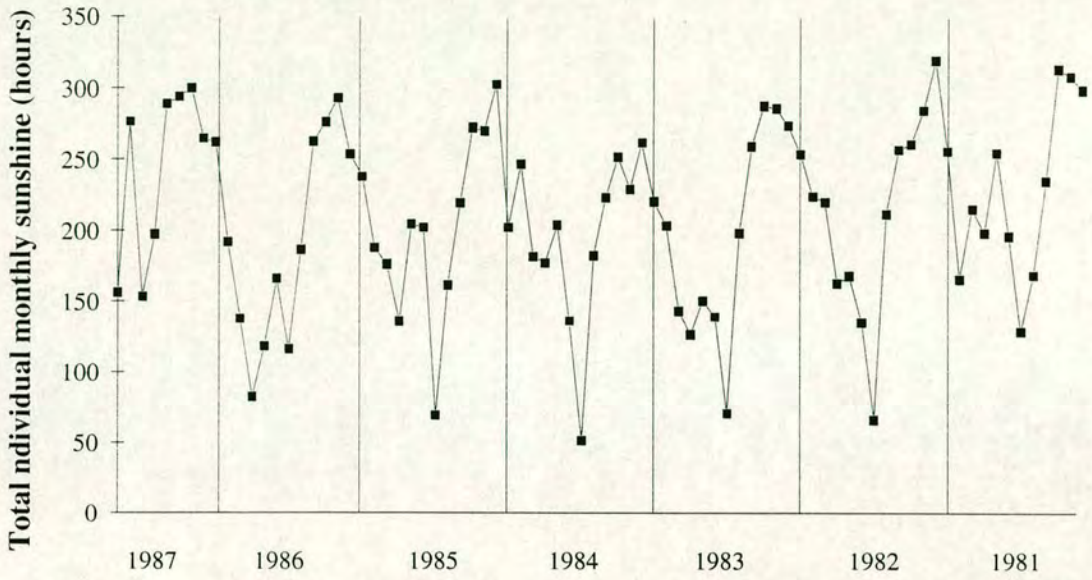


Figure 7.4 Total individual monthly sunshine (hours) between January 1981 and August 1987. Data provided by Vudichai (unpublished) working at the Phuket Marine Biological Centre. Periods of increased sunshine occur during the dry season when cloud cover is at a minimum in this area.

Thus, the effects of u/v and visible light on coral fluorophores are unlikely to be the main control on the timing of fluorescent banding in the study area.

7.2.4 The supply of organics during the wet season: Another speculative hypothesis revolves around possible variations in the coral fluorophore to total available organic mater[2] ratio of seawater through the year. If the coral incorporates organic matter from seawater into its skeleton at approximately the same rate throughout the year, and if the ratio of coral fluorophores to total available organic matter increases in the dry season (for some, at present, unidentified reason), then bright bands could be deposited during the dry season. Evidence to support this hypothesis was provided by Mopper and Schultz (1993) who categorised marine dissolved organic matter into two fractions: (1) a chemically/photochemically resistant fraction consisting of relatively young material of recent biological origin (Druffel *et al* 1989a), and (2) a chemically/photochemically labile fraction consisting of biologically utilizable material (e.g. simple sugars and amino acids); and biologically refractory material (e.g. humic acids). Although the

[2]Total available organic matter refers to any organic matter that will be taken up by the coral polyps.

chemically/photochemically resistant material is particularly abundant in the upper few hundred meters of the water column, work by Williams *et al* (1993) led Mopper and Schultz (1993) to suggest that the abundance of this material in surface waters varies seasonally. As the *Porites lutea* used in this thesis were collected from waters depths no deeper than 4 m, the seawater surrounding them should be enriched in the chemically/photochemically resistant organic fraction, especially during the wet season. Thus, even though the concentration of fluorophores in seawater may be higher during the wet season as the rains flush more terrestrial material into the marine environment, the concentration of other organic molecules may have increased by an even greater amount thus effectively diluting the fluorophore concentration in relative terms. A situation like this could arise because the soil is more frequently flushed with rainwater during the wet season, the net effect of which could be that terrestrial fluorophores have less time to form. In the dry season, terrestrial fluorophores may have more time to form so that when they are washed into the marine environment (which they will be because it does still rain even in the dry season), they will represent a higher proportion of the total organic matter. Numerous attempts were made to measure the total organic content of bright and dull bands in *Porites lutea* so that its ratio with respect to humic acid could be estimated. However, the problems associated with the complete removal of carbonate prevented meaningful results from being obtained. In addition, the reverse situation would have to occur on the Great Barrier Reef. In other words, the ratio of fluorophores to total organic matter would have to be higher in the wet season than the dry season as bright bands correlate with periods of increased rainfall in this region (Boto and Isdale 1985).

7.2.5 Summary/Key points: The fact that bright bands decrease in thickness and possibly intensity with increasing distance from the shore in the Thai study area (see Chapter 1, Figure 1.4, page 8), the Great Barrier Reef (Boto and Isdale 1985), Papua New Guinea and Indonesia (Scoffin *et al* 1989), strongly suggests that a terrestrial source is involved in bright band fluorescence. In addition, this argument is further strengthened by the discovery that local surface soil contains a high concentration of coral-like fluorophores. Bearing this in mind, none of the models discussed in this section could adequately account for the deposition of bright bands in the dry season. Thus, it is conceivable that fluorescent banding is not directly related to the supply of terrestrial fluorophores into the marine environment.

7.3 FLUORESCENT BANDING WITHOUT THE NEED FOR SEASONAL VARIATIONS IN THE SUPPLY OF TERRESTRIAL FLUOROPHORES INTO THE MARINE ENVIRONMENT:

Possible mechanisms for the production of bright bands during the dry season in this section have been divided into those in which a terrestrial source can be involved, and those that exclude a terrestrial source altogether.

7.3.1 Growth rate variations: In Chapter 5, yellow/orange fluorescent patches were discovered in *Porites lutea* using a fluorescence microscope. The predominance of these patches in bright bands suggested that they could be the cause of bright band fluorescence. If it is assumed that the rate at which fluorophores are incorporated into the skeleton remains constant throughout the year, then a faster calcification rate could result in dull band deposition, as the fluorophores would effectively be diluted by more carbonate. In addition, this model also explains why bright bands have a higher concentration of fluorophores/g of coral than dull bands i.e. bright band fluorophores are not diluted by as much carbonate.

In order to determine whether calcification rate variations occur seasonally in *Porites lutea* from Ko Phuket, it was necessary to determine both linear extension rate and bulk density, as calcification rate is calculated by multiplying these two together. Coral linear extension rates have been measured in a number of ways in the past. For example: (1) Charuchinda and Hylleberg (1984) tagged small branches of *Acropora formosa* with plastic coated wire about 3 cm from the tips and measured monthly extension rates; (2) Scoffin and Tudhope (unpublished) used alizarin red stain to estimate annual linear extension rates in *Porites lutea*. However, as this project was based in the UK, the method of Barnes and Lough (1993) was used. They estimated linear extension rate by measuring the gap between dissepiments which they assumed were deposited at approximately equal time increments. Reef front *Porites lutea* from Ko Phuket were chosen because their growth was unlikely to be limited by sea level. Coral cores were collected in July 1993 from Porites Bay on the NE shore and Shark Bay predominantly on the SW shore using a Black and Decker power drill. The cores were taken from the top of the coral colony as this is where the linear extension rate was likely to be at a maximum (see Figure 7.5).

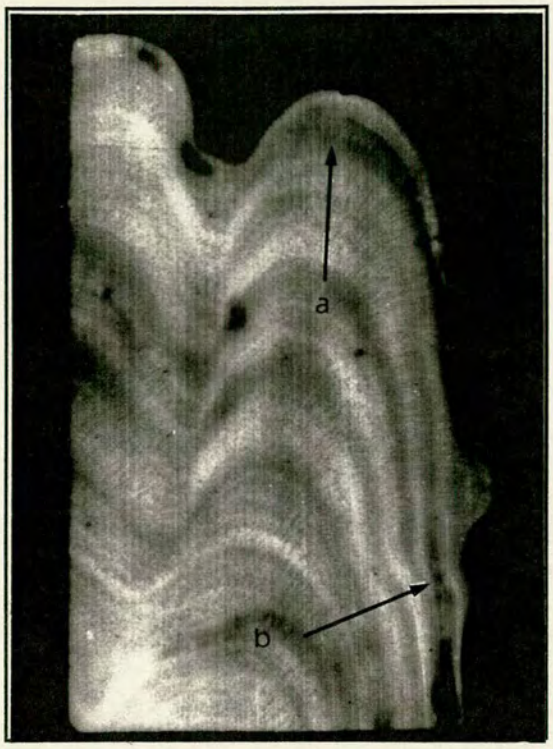


Figure 7.5 Shows how linear extension rate varies in different parts of the coral from a maximum at the top of the coral to a minimum at the sides. (a) indicates the axis of maximum linear extension while (b) indicates the axis of minimum linear extension.

Cleaned coral cores were cut perpendicular to the axis of maximum linear extension (a slow saw was used to minimise damage) and then polished with 600 grade carborundum paper. It was very important to produce sections that were truly longitudinal as deviations away from this would result in the real distance between adjacent dissepiments being over estimated (see Figure 7.6).

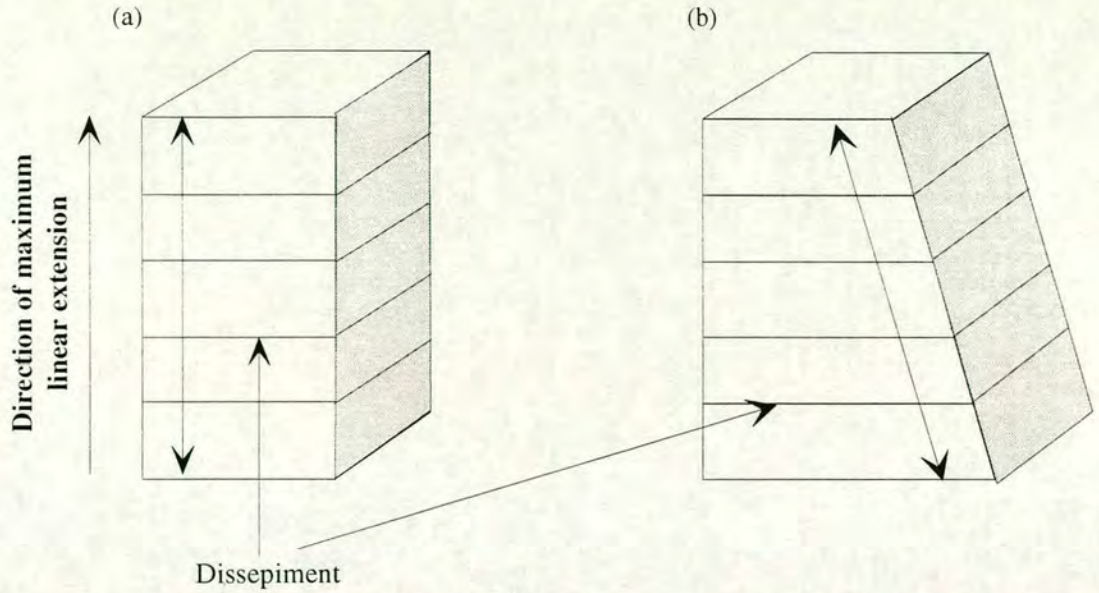


Figure 7.6 True and apparent distance between dissepiments. (a) When the coral is cut parallel to the axis of maximum linear extension, the shaded face will enable the real distance between dissepiments to be determined. (b) When the coral is not cut parallel to the axis of maximum linear extension, the shaded face will not enable the real distance between dissepiments to be determined (i.e. it will give the apparent distance between dissepiments).

Thus, only samples in which corallite walls were straight and continuous for 3-4 cm were used. The position of the fluorescent bands were then recorded on tracing paper so that they could be marked on to photographs at a later stage. Before thin sections were made, the freshly cut longitudinal surface of the coral core was impregnated with epoxy resin to minimise dissepiment damage during the thin section making process. Once made, the thin sections were photographed using a Wild Heerbrugg Photomakroskop M400 and PanF 50 film. Photographs were enlarged to enable the dissepiments to be clearly seen, and the position of the fluorescent boundaries were then marked on the prints. It is important to realise that dissepiments which form during the deposition of one particular fluorescent band, may actually be deposited in an earlier formed band i.e. in the one below. In Figure 7.7, it can be seen that although dull band material is being deposited at the coral surface, the next dissepiment will be deposited in a bright band as this is where the base of tissue layer is located. In order to account for this, the depth from the coral surface to the first dissepiment in each sample was used as the offset value. Even though Barnes and Lough (1992) found that polyp tissue thickness was not constant through the year, no seasonal variations were identified. For this reason, the depth to the first dissepiment was assumed to be constant through time.

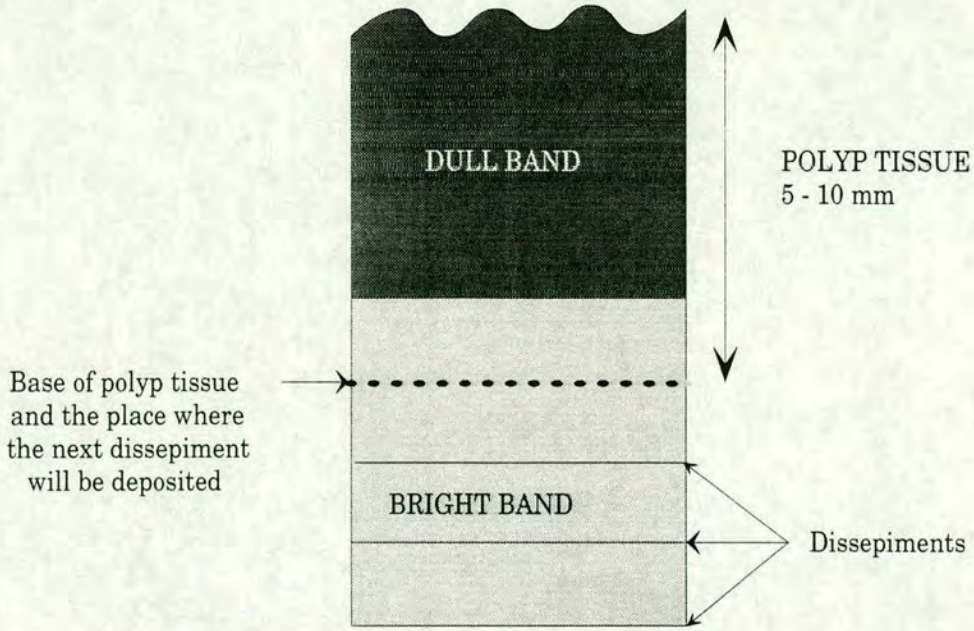


Figure 7.7 Schematic diagram demonstrating how the polyp tissue layer can span both bright and dull fluorescent bands. Although the polyp is depositing a bright band, it will deposit the next bright band dissepiment in a dull band.

The distance between dissepiments was then measured along ten vertical transects which were 1.2 mm apart. Thus, a number of measurements for the distance between any two dissepiments in a particular coral were produced. These were then averaged to give the mean dissepiment spacing.

Table 7.1 Average dissepiment spacing (mm) in bright and dull bands from Tin Smelter reef, Porites Bay and Shark Bay.

Location	DB1 (n)	BB2 (n)	DB2 (n)	BB3 (n)
Porites Bay Reef (PB-93)	1.97 (5)	1.27 (9)	1.68 (10)	1.35 (8)
Shark Bay Reef (SB-93)	1.76 (4)	1.40 (8)	1.74 (4)	1.18 (5)

Key: DB = Dull bands; BB = bright bands; (n) = the number of dissepiments within the band once the tissue layer thickness correction factor had been applied. Bands are numbered in relation to their closeness to the living coral surface. For example, DB1 is the nearest dull band to the living coral surface. As dissepiments are not found within the tissue layer, and as the outer most bright band was not fully formed, the first band analysed was DB1 while the first bright band analysed was in fact BB2 rather than BB1. The number of dissepiments can be seen to vary between corals and between bands within the same coral. This could reflect the fact that fluorescent bands do not necessarily represent equal periods of time i.e. a couplet may represent 10 months one year and 14 months the next.

Results in Table 7.1 show that dull band dissepiment spacing is greater than bright dissepiment spacing. These variations could reflect seasonal variations in linear extension rate with dull band linear extension rate being greater than bright band linear extension rate. These results are supported by visual observations. For

example, Figure 7.8 shows a coral from Tin Smelter Bay that was stained with alizarin red in November 1986 (at the end of the wet season) and collected in August 1987.

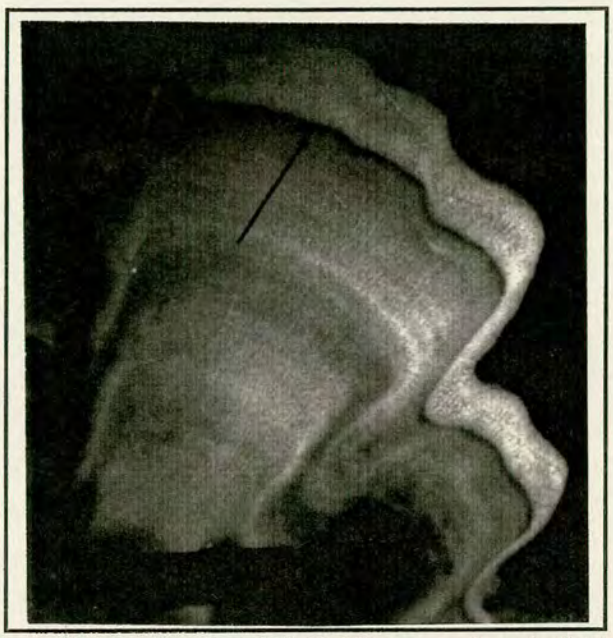


Figure 7.8 A coral from Tin Smelter Bay (TS-25-87) seen under u/v light. The scale bar is 5 cm long. The November 1986 stain is labelled.

It can be seen that in the nine months after the stain was deposited, bright band material was deposited exclusively. In the year before the stain was deposited, the linear extension rate was approximately 2.8 cm/year. Assuming that linear extension rate is moderately constant between years, the coral could be expected to grow by a similar amount in the year after the stain. In the nine months after the stain, the coral grew approximately 1.7 cm, in which case, during the remaining three months (i.e. during dull band deposition), it would be expected to grow approximately 1.1 cm. In other words, the coral has to grow 40% in 25% of the time, which suggests that dull band linear extension rate is greater than bright band linear extension rate.

If this is the case, then these results are in contrast to those of Chansang *et al* (1992) who reported higher linear extension rates in *Porites lutea* during the dry season. However, the *Porites lutea* used by Chansang *et al* (1992) were collected from sites on the western coast of Ko Phuket which were ~20 km away from the reef sites sampled in this thesis. Although the reef sites used in their study and those on the SW shore of the peninsula in this thesis were exposed to the SW Monsoon, the latter were not exposed to the full force of this Monsoon (the corals on the NE shore of the

peninsula were not exposed to the SW Monsoon at all). Thus, the opposing relationship suggested by results in Table 7.1 between linear extension rate and season could be related to relative exposure to the SW Monsoon.

The average bulk density of corals growing at Porites Bay and Shark Bay were obtained from Chapter 2 (see Table 2.1, page 31). The mean bright band bulk density in Porites Bay was 0.98 g/cm^3 while the mean dull band bulk density was 0.93 g/cm^3. The mean bright band bulk density in Shark Bay was 1.12 g/cm^3 while the mean dull band bulk density was 1.18 g/cm^3. To determine linear extension rate/year, the mean gap between dissepiments for a particular band was multiplied by the number of dissepiments deposited in a year. Barnes and Lough (1993) have shown that the number of dissepiments deposited in a year in *Porites spp.* varies between 10.2 and 11.9 (i.e. slightly less than one dissepiments deposited per month). Although their readings range from as low as 3.4 dissepiments/year, they never exceed 12.4. As there was insufficient data to determine the average number of dissepiments deposited/year in *Porites lutea* from Ko Phuket, the Barnes and Lough (1993) data was used. Thus, linear extension rate/year was determined by multiplying the mean gap between dissepiments by 11 (i.e. the mid point between 10.2 and 11.9). Calcification rate was then determined by multiplying bulk density and linear extension rate.

Table 7.2 Calcification rate calculations.

PB-93:		**SB-93:**	
Band	CR	Band	CR
DB1	2.02	DB1	2.28
BB1	1.37	BB1	1.72
DB2	1.71	DB2	2.26
BB2	1.46	BB2	1.46
Mean DB	**1.87**	Mean DB	**2.27**
Mean BB	**1.41**	Mean BB	**1.59**

Key: CR = calcification rate: all units given as g/cm^2/year.

Bearing in mind the limited amount of data and the fact that the number of dissepiments deposited per year in the Thai corals was not determined, the results shown in Table 7.2 nevertheless suggest that dull band calcification rate is higher than bright band calcification rate in *Porites lutea* from Porites Bay and Shark Bay.

To summarise, evidence in Table 7.2 supports the hypothesis that an increase in the rate of calcification could result in dull band deposition in the study area regardless of origin of the yellow/orange fluorescent patches.

7.3.2 Do terrestrial fluorophores really play a part in coral fluorescence? This is a valid question considering fluorescent banding is seen in *Porites* from Oman where there is essentially no terrestrial input (see Chapter 1, Figure 1.6, page 11). Although terrestrial fluorophores could have travelled there, the effects of photo-oxidation (Kouassi and Zika 1990), and the observation that terrestrial humic acids quickly flocculate when reaching the marine environment (Sholkovitz 1975), suggests that terrestrial humic acids are unlikely to travel far from the mainland. The fact that fluorescent banding is seen in *Porites lutea* from the Similan Islands (70 km offshore) may be attributed to the fact that the Similan Islands can support plant and animal life (i.e. the fluorophores are probably generated locally rather than from the mainland).

If terrestrial fluorophores do <u>not</u> play a significant part in coral fluorescence, then the coral fluorophores must be derived from other sources. The two most likely being those generated *in situ* (i.e. by the polyp or zooxanthellae) and/or those generated in the marine environment by planktonic organisms (e.g. *Trichodesmium*). Thus, although non-terrestrial fluorophore sources do exist, a mechanism that results in a greater concentration of fluorophores during the dry season is still required.

Primary productivity variations: In areas which are free from terrestrial input but where corals are actively depositing seasonal fluorescent bands (i.e. bright and dull) such as in Oman, bright band deposition could coincide with peaks in primary productivity. Certainly, when primary productivity data (obtained from the Phuket Marine Biological Centre, Vudichai, unpublished) was plotted between August 1981 and December 1986, peaks in primary productivity quite often occurred during the dry season. Such evidence could be used to suggest that there is a link between primary productivity and bright band deposition. The fact that peaks in primary productivity can be caused by a number of factors acting either independently or together (e.g. seawater temperature, light availability, nutrient supply, salinity) is probably the reason why the monthly record of primary productivity is so variable. The predominance of peaks during the dry season could be due to increased light (and hence increased photosynthesis), but it could also be due to coastal upwelling. In the Oman area, this coastal upwelling is known to occur during the SW Monsoon

and brings nutrient-rich water to the surface; this then results in a primary productivity bloom and possibly an increase in seawater humic acid concentration and the deposition of a bright band. Although no data has been published on coastal upwelling in the Thai area, Vudichai (unpublished) has recorded a slight drop in seawater surface temperature off the edge of the continental shelf to the south of the Similan Islands during the dry season. This drop in surface temperature could be due to coastal upwelling and may result in the deposition of bright bands during the dry season (i.e. the NE Monsoon).

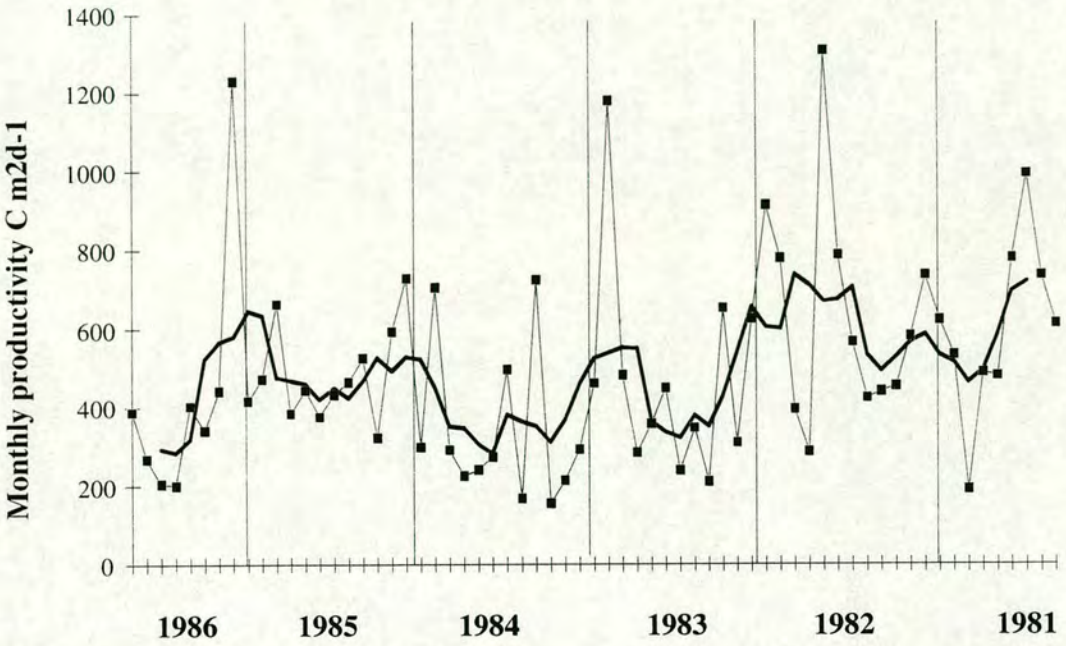


Figure 7.9 Productivity data. Data obtained from the Phuket Marine Biological Centre from August 1981 to December 1986. Productivity data were determined at the Phuket Marine Biological centre (unpublished) and are expressed as mg C m^{-2} d^{-1}. The solid black line represents the five month average monthly productivity values.

However, before one links primary productivity with bright band deposition, the following points have to be considered:

(a) Only a very limited amount of data were available. A much larger data set would be required before it would be possible to say with certainty whether there is any seasonality in primary productivity.

(b) A peak in primary productivity does not necessarily mean a peak in seawater fluorophore concentration as marine fluorophores may only make up a small percent of the primary production.

(b) A peak in primary productivity does not necessarily mean a peak in seawater fluorophore concentration as marine fluorophores may only make up a small percent of the primary production.

Although plankton are believed to be the most likely source of marine humic acids, very few specific sources have actually been identified. One such source is the blue/green marine alga *Trichodesmium*. Jones and Thomas (1989) suggested that reduced salinities (36 to 22‰) caused by freshwater runoff during the wet season result in the death of *Trichodesmium* and the release of humic acids into the marine environment. However, it is unlikely that *Trichodesmium* is the main cause of dry season bright band deposition for the following reasons: (1) although seawater salinity in Phangnga Bay does not vary significantly throughout the year (31.5 to 33.5‰: Charuchinda and Hylleberg 1984), the release of humic acids from *Trichodesmia* is more likely to occur during the wet season (due to surface water runoff) in which case a bright band might be expected at this time; and (2) No such increase in seawater humic acid concentration was detected during algal blooms of red tide proportions by Susic *et al* (1991). Thus, seasonal variations in *Trichodesmium* concentration are unlikely to be the main cause of the fluorescent banding seen in *Porites lutea* in the study area.

7.3.3 Summary/Key points: The fact that fluorescent banding is seen in *Porites spp.* from Oman suggests that fluorescent banding can be formed in the absence of terrestrial fluorophores. This does not, however, mean that terrestrial fluorophores play no part in coral fluorescence in areas where a significant terrestrial input into the marine environment is known to occur. Of the mechanisms discussed in this section which do not necessarily require a terrestrial source for fluorescent banding, the most likely scenario involves growth rate variations.

7.4 DISCUSSION/CONCLUSIONS:

(A) Although work in Chapters 4, 5 and 6 has discussed possible ways in which bright bands may arise, the reason or reasons why bright bands are deposited in the dry season in Ko Phuket were not absolutely identified. Even though variations in the rate of calcification appear to be the most plausible mechanism for bright band deposition in the dry season, there are a number of unknowns associated with this model which would have to be investigated before this hypothesis can be accepted.

For example: (1) Is the rate of fluorophore uptake constant throughout the year? If the rate varies then would the hypothesis still stand? (2) What proportion of the fluorophores are generated *in situ* rather than being ingested? If they are all generated *in situ*, then the concentration of fluorophores in the seawater will not affect the rate of fluorophore incorporation into the skeleton; (3) What is the calcification rate for corals who's bright bands are deposited in the wet season?

(B) A final, and as yet undiscussed, hypothesis, is that fluorescent banding in *Porites lutea* from Ko Phuket, and indeed other areas of the world where coral fluorescence has been studied, is a combination of more than one factor. However, before the effects of a combination of factors on coral fluorescence can be assessed, a better understanding of the effects of individual factors is required first.

CHAPTER 8. CONCULSIONS

CHAPTER 8. CONCLUSIONS

8.1 SUMMARY/KEY POINTS:

8.1.1 Why are bright bands bright?: This thesis attempted to understand more fully: (1) the processes that are involved in the production of coral fluorescence, and (2) the reason(s) why bright bands are deposited in the dry season in the Thai study area even though they are deposited in the wet season in all other areas where coral fluorescence has been studied to date. The principal conclusions can be summarised as follows:

(A) Although visual observations suggested that fluorescent banding and porosity/bulk density were directly related (i.e. the larger the pores, the brighter and lighter the fluorescence), photographic evidence (comparing density bands with fluorescent bands) and the direct measurement of bulk density and porosity, have demonstrated that variations in porosity/bulk density do not control fluorescent banding. Porosity can, however, affect fluorescent intensity as work has shown that fluorescent intensity increases with increasing porosity (and therefore decreasing bulk density). This was thought to relate to the amount of coral skeleton exposed to u/v light and hence the number of fluorophores excited.

(B) As macroporosity was shown to have an effect on coral fluorescence, the effects of microporosity (i.e. porosity between 4 μm and 100 μm) were also examined. Microporosity appears to have the ability to reduce coral fluorescence, a phenomenon which is thought to be associated with the presence of clays. However, the fact that: (1) so few microborings were found in coral skeleton that was covered by living polyp tissue at the time of collection, and (2) no significant difference was detected between bright and dull bands in terms of the number of microborings, suggested that microboring activity is not the main cause of fluorescent banding.

(C) In order to examine coral fluorescence without the compounding effects of porosity and $CaCO_3$, the fluorescent properties of coral solutions in which the $CaCO_3$ was dissolved using 1N HCl were studied. Results showed that coral fluorescence consisted of two, possibly three, fluorophore groups: a possible excitation peak group at 275-285 nm, the dominant 330-345 nm excitation peak group, and the 390 nm excitation peak group. The 330-345 nm and 390 nm excitation peak groups were

also the dominant excitation peaks in low concentration (i.e. less than 36 ppm) industrial humic acid solutions. This, therefore, suggested that humic acids were the dominant fluorophores in coral skeletons which is consistent with work by Boto and Isdale (1985) and Susic *et al* (1991). When bright and dull band coral solutions were compared, no difference in the shape of the excitation/emission (EXEM) spectra or the predicted colour of fluorescence (both blue) could be detected; the only difference between the two being one of intensity, with bright bands being more intense than dull bands. Thus, although Boto and Isdale (1985) suggested that bright band fluorescence was due to humic acids from two different sources (marine and terrestrial), results in this thesis suggested that the compounds responsible for fluorescence in both bright and dull bands could be the same, which is in keeping with work by Quale (1991).

(D) The metal ions, iron and manganese, have been shown to affect both the intensity and colour of coral fluorescence. However, they were not present in large enough quantities, and no significant variation in terms of their concentration was detected between the bands. This therefore suggested that these ions are unlikely to be the main cause of fluorescent banding in *Porites lutea* from Ko Phuket. Work involving industrial humic acid (Fluka and Aldrich) demonstrated that changes in intensity could occur as a consequence of concentration differences below the energy transfer (ET) threshold. However, above the ET threshold, increases in concentration result in both a decrease in fluorescent intensity and a shift in the colour of humic acid fluorescence to longer wavelength emissions (e.g. blue to yellow/orange). The fact that 1M coral solution fluorescence was blue, regardless of band type, suggested that the concentration of fluorophores in the solutions was below the ET threshold for both bands, hence no different in colour, only intensity. Bearing in mind that humic acids are thought to be responsible for approximately 75% of coral fluorescence (Susic *et al* 1991), industrial (Fluka) humic acid solutions were used to estimate the concentration of fluorophores in 1M coral solutions. Taking into account the large dilution factor involved when making corals solutions (as coral density can range from approximately $1g/cm^3$ to $2.94g/cm^3$ depending on whether or not the pore spaces are included in the calculations, the dilution factor could be anywhere between 10 and 30), it was possible to estimate the concentration of fluorophores in the solid state. Calculations suggested that both solid state bright and dull band fluorophore concentration (140 ppm to 420 ppm) were well above the ET threshold value associated with humic acid solutions (~36 ppm). Although the errors associated with the fluorophore estimates in the solid state are probably very high (due to the

problems of estimating fluorophore concentration using industrial humic acid solutions), both bright and dull bands should emit fluorescence at wavelengths longer than blue. However, visual observations demonstrated that this was not the case. Although this may well suggest that the estimates of fluorophore concentration in the solid state are inaccurate, an alternative explanation lies in the manner in which the fluorophores are distributed within the skeleton.

(E) When solid coral was examined using a fluorescence microscope, the fluorescence seen could be divided into two main components: (1) a dominant (in terms of area) blue background fluorescence, and (2) randomly distributed yellow/orange fluorescent patches (8 μm to 35 μm). When these areas were examined under transmitted light conditions, the fluorescence seemed to originate from relatively small (5 μm to 6 μm), dark, possibly organic, inclusions. Emission spectra from the yellow/orange patches were very similar to the humic acid emission spectra presented by Boto and Isdale (1985), which therefore suggests that these dark inclusions were humic acids or something similar. Although the origin and mechanism of incorporation of these inclusions was not absolutely identified, the fact that yellow/orange fluorescence is seen is, therefore, strong evidence in favour of a colour difference (in addition to an intensity difference) between bright and dull bands.

(F) The colour of the fluorescence seen is thought to be due to the ratio of yellow/orange patches to background fluorescence. Although coral fluorescence is dominated by emissions that are essentially purple in colour in both bands, fluorescent bands are certainly not different shades of purple. The reason why fluorescent bands are not the colour of the dominant emission wavelength is thought to be due to the eyes' response to different wavelengths of light (i.e., even though short wavelengths may dominate, the eye is much better at seeing yellow/orange light than purple/blue light). Thus, even though the yellow/orange patches are relatively rare, they will have a large effect on the colour seen. The (apparently) larger number of yellow/orange patches in bright bands, could therefore be the cause of any colour difference between the two band types. However, when solid state EXEM spectra were produced, no significant difference in terms of the colour of fluorescent emissions was detected between bright and dull bands. This was, however, probably due to the photomultiplier (PMT) used which could not record emissions above 650 nm and its response to emissions between 580-650 nm was very poor.

(G) The fact that differences in colour were not detected instrumentally between bright and dull band coral solutions could also be attributed to the PMT. However, and more significantly, it is probably because the fluorophores in the high concentration yellow/orange patches dissolve and become evenly distributed throughout the solution. Although the concentration of fluorophores in the yellow/orange patches is above the ET threshold, they are so few in number that when they dissolve and mix with the background fluorophores, the increase in concentration produced in the solution is minimal. Another reason why fluorophore concentration in solutions is below the ET threshold is because a large dilution factor (estimated at between 10 and 30) is involved when making 1M coral solutions.

(H) If fluorescence with longer wavelengths than blue is to be emitted, the concentration of fluorophores must be above the ET threshold. However, results using the LS-5 spectrofluorimeter and the fluorescence measuring device both suggested that shorter wavelengths dominated. In other words, the concentration of fluorophores in the solid state was below the ET threshold, irrespective of band type. While this is probably the case in the skeleton as a whole, the concentration of fluorophores is thought to be above the ET threshold in the yellow/orange fluorescent patches which is why they are that colour. However, the fact that fluorescence is dominated by emissions between 400 nm and 500 nm, probably reflects the minor contribution made by the yellow/orange fluorescent patches to the total fluorescence emitted by the coral. As variations in fluorescent intensity reflect variations in fluorophore concentration below the ET threshold, bright bands would appear to have a higher concentration of fluorophores than dull bands as they are, in general brighter. Thus, because of the way in which the fluorophores are distributed in the skeleton, fluorescent banding would appear to be both a colour and an intensity phenomenon in the solid state.

(I) Coral fluorescent intensity is probably controlled by the combined effects of yellow/orange fluorescence and background fluorescence. The fact that short wavelength emissions dominate suggests that background fluorescence is the main control on fluorescent intensity. As the concentration of fluorophores in the background region of the skeleton would appear to be below the ET threshold for both bright and dull bands, the intensity of the fluorescence emitted is a good indication of the concentration of fluorophores in the skeleton as a whole. As bright bands are in general more intense than dull bands, this tends to suggest that the concentration of fluorophores in bright bands is higher than in dull bands.

(J) Attempting to determine the source or sources of coral fluorophores was not greatly assisted by the discovery of coral-like fluorophores in polyp tissue, seawater and nearby surface soil. Although the discovery of a 275-285 nm excitation peak group in coral solutions could be used as proof of a marine source (this peak was not found in terrestrial samples), it could also be due to isoxanthopterin (a cell metabolite) and thus produced *in situ*. The fact that bright band thickness, and possibly intensity, decreased with increasing distance from the shore (in Ko Phuket), combined with the discovery that the concentration of fluorophores in soils/sediments also decreased with increasing distance from the shore, could indicate a terrestrial source for bright band fluorescence. However, the absence of a terrestrial source in Oman where fluorescent banding is seen in *Porites spp.* suggests that terrestrial fluorophores are not essential for fluorescent banding to occur.

8.1.2 Why are bright bands deposited in the dry season in the Thai study area?: There are a number of reasons why it was not possible to correlate the fluorescent profiles produced using the fluorescence measuring device with the environmental parameters, rainfall, sunshine and productivity. These can be listed as: (1) the unexplained trends towards increasing and decreasing fluorescent intensity with increasing distance from the living coral surface which could be due to any of a number of factors, for example: microbial action, ageing of the fluorophores, photo-oxidation, pore orientation; (2) coral fluorophores appear to be heterogeneously distributed throughout the skeleton which means that readings from adjacent areas in the same band could give very different readings; (3) although the longest record spanned approximately seven years, the other records were only three to three and a half years long, (i.e. the records were fairly short); (4) master curves were not created for each colony (the point of which is to reduce the natural variability produced by the heterogeneous distribution of fluorophores and pore spaces). Thus, it was not possible to confirm or refute suggestions made by previous authors that rainfall and fluorescence are positively related. Although a variety of mechanisms were proposed for the deposition of bright bands during the dry season, the most likely one involved variations in calcification rate. Results showed that in the Thai study area, calcification rate was faster during the deposition of dull bands. Thus, assuming the uptake of fluorophores was constant throughout the year, this would result in the concentration of fluorophores being diluted by more carbonate during the wet season. Although such a hypothesis works equally well in the absence of a terrestrial input, the rate of fluorophore uptake would have to be determined before this hypothesis can gain some acceptance.

8.2 RECOMMENDATIONS FOR FUTURE WORK:

(A) As corallites in *Porites lutea* are between 1 to 1.5 mm in diameter, the excitation strip was unable to analyse individual corallite pore spaces. Thus, it was not possible to determine whether fluorescent intensity was greater in pore spaces or whether the colour of fluorescent emissions was any different to non-pore spaces. Although a smaller beam could be produced by using a diaphragm, a more intense u/v source may be required to obtain a sufficient intensity to measure fluorescent emissions. Alternatively, coral with a larger diameter corallite could be used such as *Goniastrea aspera* which has a corallite diameter of between 7 mm and 10 mm. Such work would help determine how pore space orientation affects fluorescent profiles.

(B) One of the questions not answered in this thesis was to what extent marine and terrestrial humic acids were involved in coral fluorescence. The fact that no significant difference could be detected between the EXEM spectra of bright and dull band solid corals and coral solutions suggested that the compounds responsible for fluorescence were more or less the same. If fluorescent banding in *Porites lutea* is due to the presence of humic acids ingested from the marine environment (rather than generated *in situ*), then either marine and terrestrial humic acids are very similar, or only one of these are involved in fluorescent banding. It may be possible to answer this question in the following ways: (1) Obtaining a known mass of marine (must be far removed from terrestrial source) and terrestrial humic acids. By making solutions of equal concentration it may be possible to detect whether there is a difference in their EXEM spectra, and also whether their fluorescent efficiencies are the same; (2) As Nissenbaum and Kaplan (1972) have reported that marine and terrestrial humic acids have different ^{13}C:^{12}C ratios (marine humic acid ^{13}C values vary between -20 and -22‰ and terrestrial humic acid $\delta^{13}C$ values vary between -25 and -26‰), bright and dull band $\delta^{13}C$ values could be determined to see the contributions made by each component.

(C) Although EXEM spectra are a great improvement on emission spectra, specific sources were not immediately obvious. Future work involving the examination of soil and sediment extracts and coral solutions cooled by liquid nitrogen may help to increase excitation peak resolution possibly enabling specific fluorophore sources to be identified. This is because large molecules, such as humic acid, have many vibrational levels/states which give rise to broad spectra. Cooling the molecules

down reduces the number of these vibrational levels that are populated thus resulting in sharper spectra.

(D) Much has been made of the involvement of yellow/orange fluorescent patches in producing a colour difference between bright and dull bands. However, due to time constraints, no hard data was collected to back up visual observations. The ratio of yellow/orange fluorescent patches to background fluorescence could be determined by taking a large number of photographs along a known path in both bright and dull bands and then point counting.

(E) To enable coral fluorescence to be compared to environmental parameters such as rainfall, sunshine and productivity, a better understanding of the reason or reasons for the trends towards increasing or decreasing fluorescent intensity with increasing distance from the living coral surface is required. This could be achieved by: (1) taking longer core sections so that a larger amount of data are available, and (2) taking a number of transects from one coral, averaging them (thus reducing local anomalies cause by surface preparation, corallite orientation, fluorophore distribution), to create a master curve for that core. This could be done for two or three cores from the same colony and a master curve produced for that colony. Profiles produced in this way could be compared with similar profiles produced for other colonies.

(F) A substantial program of water sampling (i.e. weekly throughout the year) should be undertaken for the following reasons: (1) to see how the concentration of fluorophores in the seawater changes in response to the onset of the seasons (i.e. to see if there is a lag effect); and (2) to monitor changes in primary productivity and humic acid concentration.

(G) Because coral fluorescence has been observed in areas free from a terrestrial input, the question 'do terrestrial humic acids play a part in coral fluorescence' arose. In order to answer this question, I would like to do the following: (1) sample more corals in such areas whilst monitoring seawater humic acid levels to see if phytoplankton blooms (possibly related to coastal upwelling or some other phenomenon) coincide with bright band deposition; (2) grow a coral in a humic acid free environment to see whether (a) humic acids are essential for the survival of the polyp and/or for skeletogenesis, and (b) whether fluorescence changes.

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APPENDIX 1: INTRODUCTION

A1.1 Humic substances: The term "humic substance" describes a general category of compounds which have been defined as naturally occurring, biogenic, heterogeneous organic substances (Aiken *et al* 1985). Humic substances are commonly divided into three classes on the basis of solubility (Thruman *et al* 1988):

(a) **Humins:** the fraction of humic substances not soluble in water at any pH.

(b) **Humic acid:** the fraction of humic substances not soluble in water at pH 1 or pH 2 but soluble at greater pH.

(c) **Fulvic acid:** the fraction of humic substances soluble under all pH conditions.

This system of classification has continually been modified as various authors prefer different definitions. For example, Boto and Isdale (1985) defined fulvic acids as the fraction of humic material soluble at pH 1 (rather than 1 or 2). Susic and Boto (1989) defined humic acids as the alkali soluble extract from soils and fulvic acids as the acid soluble fraction of these extracts. As mentioned in the main body of the text, this thesis makes no effort to differentiate between humic and fulvic acids and uses the term humic acid exclusively. This is because work by Susic *et al* (1991) suggested that there was no significant chemical difference betwen the two in solution.

The formation of humic substances: Humic substances have been detected in most natural environments including soils, sediments, lake water and seawater. Their wide distribution suggests that either many precursors and formation pathways exist, or conditions favouring a limited number of formation mechanisms are widely found in nature. However, it is still a matter of debate as to whether humic substance formation occurs metabolically, to form biopolymers which are then marginally altered to humic substances, or, takes place in the later stages of biopolymer decomposition as small reactive decomposition molecules spontaneously recombine. As a consequence of this, two main types of model for the formation of humic substances have developed:

(a) **Biopolymer degradation models**: These models assume that polymerisation occurs within cells to form biopolymers which are then partially degraded in the environment to produce humic substances. They also assume that humic acids are precursors to fulvic acids. During microbial degradation, the labile macromolecules are degraded and lost while the refractory ones, such as lignins, cutins, N-containing paraffinic macromolecules, are preserved to become humin. Evidence to support such models comes from the identification of lignin-like material in humic acids (Hatcher *et al* 1985). The greatest appeal of biopolymer degradation models is their assumption that the framework of humic substances is assembled by living organisms via enzymic mediation. In this way, large molecules can be constructed without requiring: (1) a high concentration of precursors, and (2) labile precursors, both of which are generally necessary for extra-cellular polymerisation. Although Waksman's lignin protein model (1932) for the formation of soil humic substances via the partial degradation of lignin polymers has probably been the most accepted biopolymer degradation model, lignin is a material found in land plants only. In marine sediments or sediments dominated by inputs from non-vascular or non-lignin containing plants, it is difficult to envisage lignin as being a major player in humic substance formation.

(b) **Abiotic condensation models**: These models involve the condensation (or repolymerisation) of small reactive organic molecules. Abiotic condensation models are much harder to test as the starting point and mechanisms of formation are much less well defined. The polyphenol model, popularised by Kononova (1966), is the most commonly cited abiotic condensation model and assumes that phenols polymerize to produce quinones which in turn polymerise to form first fulvic and then humic acids. One of the strengths of this model is that simple phenols are common microbial degradation products of lignin (Kirk 1984), and are known to be synthesised by fungi of the *Imperfecti* group (Martin and Haider 1971). In natural environments, the oxidation of polyphenols to quinones can either occur spontaneously in the presence of oxygen, or, be enzymatically controlled by a wide variety of organisms (Stevenson 1982). Since many micro-organisms have difficulty degrading aromatic substances, phenols have a good chance of persisting in high enough concentrations to be effective quinone precursors. Quinones readily polymerise with each other or amino acids and ammonia to form synthetic polymers (Stevenson 1982) which have bulk chemical properties that closely resemble natural humic substances

(Ladd and Butler 1966; Ertel and Hedges 1983). There are however, a number of problems associated with the polyphenol model. Although present, phenols are generally not abundant in phytoplankton which are the major producers of organic matter in aquatic environments. In addition to this, the oxidation of phenols to quinones is difficult under the suboxic conditions encountered in marine sediments.

Both of these models suggest that humic substances are generally of terrestrial origin. Although it was originally assumed that humic substances in marine sediments were allochthonous (i.e. transported there from a terrestrial source) (Degens *et al* 1964), plenty of evidence now exists in favour of a marine source of humic substances. For example, Sieburth and Jensen (1968), Sholkovitz (1975) and Sholkovitz *et al* (1977) demonstrated that humic materials from continental sources rapidly precipitate on entering the marine environment and therefore, humic acids found in the marine environment far from a terrestrial source must be marine in origin. Perhaps the most convincing evidence of a marine source of humic substances was provided by Nissenbaum and Kaplan (1972) who demonstrated that the nitrogen content and carbon isotopic composition of humin from marine and terrestrial sediments were quite distinct. They also suggested that plankton are the most likely source of marine humic substances.

APPENDIX 2: THE RELATIONSHIP BETWEEN FLUORESCENT BANDING AND PORE SPACES

A2.1 Determining the precision of the bulk density measurements: Measurements were taken from PB-4-87. The bulk density was determined by dividing the mass of a particular sample (the mean of eight separate measurements), by its volume (also the mean of eight separate measurements).

Table A2.1 Precision of bulk density measurements

Mass (g)	Mean bulk density (n=8)	STDEV	% error
3.47	1.169	0.001	0.106
1.62	1.129	0.002	0.186
1.64	1.169	0.001	0.083
0.70	1.116	0.002	0.137
0.74	1.146	0.001	0.108
0.66	1.118	0.001	0.083
0.71	1.146	0.001	0.127
0.14	1.110	0.002	0.160
0.14	1.103	0.002	0.171
0.10	1.083	0.004	0.372
0.09	1.123	0.003	0.277
Mean percentage error of all samples			**0.165**
Mean percentage error of all samples heavier than 0.25 g			**0.119**

Key: STDEV = standard deviation, % error = the standard deviation as a percentage of the mean.

Results in Table A2.1 show that the measurements of bulk density are very precise. This of course does not necessarily mean that these figures are accurate, a topic that is discussed in the main body of the text.

A2.2 Determining whether there is a relationship between bulk density and sample mass: In order to determine statistically whether there is a relationship between bulk density and sample mass, the slope (or gradient) of a best fit straight line plotted through a series of points was calculated using bulk density measurements collected from two bright bands (BB1 where n = 14; and BB2 where n=14) and one dull band (DB1 where n=16) from AQ-1-88. The gradient was calculated using the following formula:

$$b = \frac{N.\sum(x.y) - \left[\sum(x).\sum(y)\right]}{N.\sum(x^2) - \left(\sum(x)\right)^2}$$

where: b = slope of the line; N = number of samples; x = mass (g), y = bulk density (g/cm^3). All samples had a mass greater than 0.25 g. It can be seen in Table A2.2 that in all three cases, the slopes are slightly positive (i.e. bulk density decreases with decreasing sample mass).

Table A2.2 Calculating the slope of a best fit line that is produced when bulk density and sample mass are plotted against each other.

Band	Slope b	95% CI	95%	-95%	Status
BB1	0.0041	0.0010	0.0051	0.0031	Positive slope
BB2	0.0079	-0.0009	0.0070	0.0088	Positive slope
DB1	0.0026	0.0014	0.0040	0.0012	Positive slope

Key: CI = confidence interval.

To determine whether the gradients were significantly different from zero, a Pearsons correlation coefficient (r) was calculated using the following formula:

$$(r) = \frac{Cov(x.y)}{\sqrt{\left[Var(x).Var(y)\right]}}$$

where:

(r) = Pearsons correlation coefficient
Cov (x.y) = Covariance of x.y
x = Mass (g)
y = Bulk density (g/cm^3)
Var = Variance

Results in Table A2.3 show that the (r) values are very close to zero which suggests that there is no relationship between bulk density and sample mass. This was confirmed when a students 't' test was performed[1] (also see Table A2.3).

Table A2.3 Determining whether there was a relationship between sample mass and bulk density.

Pearsons correlation coefficient (r):

Band	Slope	(r)
BB1	0.0041	0.1935
BB2	0.0079	0.1483
DB1	0.0026	0.0080

Students 't' test:

	't' test	Confidence interval	Critical value
BB1	0.68	99%	3.012
		95%	2.16
		90%	1.771
BB2	0.52	99%	3.012
		95%	2.16
		90%	1.771
DB1	0.03	99%	2.947
		95%	2.131
		90%	1.753

Key: (r) Pearsons correlation coefficient. Values of (r) range between -1 and +1. When (r) equals zero, no relationship exists.

[1] A students 't' test was used to see if the calculated value of 'r' differed significantly from zero.

A2.3 Porosity measurements: Porosity measurements were obtained directly from thin sections which were approximately 10-15 mm long and 5-10 mm wide. Thin sections were made from the coral blocks used in Chapter 2, section 2.3. In order to determine whether porosity varied according to the type of section taken (i.e. longitudinal or transverse), six longitudinal and six transverse thin sections were point counted using AQ-1-88 (see Table A2.4).

Table A2.4 The porosity of longitudinal versus transverse sections.

TS	PS %	LS	PS %
1	52.93	7	54.26
2	52.07	8	51.12
3	55.16	9	58.48
4	54.47	10	55.28
5	50.45	11	53.06
6	54.93	12	56.28
Mean	**53.33**	Mean	**54.75**
STDEV	1.86	STDEV	2.56
% error	3.48	% error	4.68

Key: TS = transverse section; LS = longitudinal section; % PS = percent pore space, STDEV = standard deviation, % error = percentage error (calculated as the STDEV as a percentage of the mean).

A Mann-Whitney U-Test was then performed to determine whether the difference shown in Table A2.4 between TS and LS porosity was significant. Results suggest that this difference is insignificant, even at the 90% confidence level (see Table A2.5). This was not altogether surprising considering the bulk density of a block of coral is the same no matter which direction it is measured in. Thus, although one thin section might produce an anomalous porosity value, the average porosity calculated from a number of thin sections taken randomly is unlikely to suffer from this problem. Therefore, no effort was made to distinguish between longitudinal and transverse sections.

Table A2.5 Mann-Whitney U-Test

TS	PS %		Rank	LS	PS %		Rank
1	52.93		4	8	54.26		6
2	52.07		3	9	51.12		2
3	55.16		9	10	58.48		12
4	54.47		7	11	55.28		10
5	50.45		1	12	53.06		5
6	54.93		8	13	56.28		11
		Sum	32			Sum	46

U1	25
U2	11

Critical values for a two tail test are given below:

99%	2
95%	5
90%	7

Key: TS = transverse section; LS = longitudinal section; % PS = percent pore space.

To make it easier to distinguish pore space from skeletal material, samples were impregnated with dyed blue araldite before they were thin sectioned. Because of the porous nature of *Porites lutea*, it is very difficult to get the araldite to fill every pore space even when impregnation is carried out under vacuum (i.e. air bubbles form). As their exclusion would artificially reduce porosity, air bubbles were counted as pore spaces. Porosity was estimated by point counting under reflected light. Because both dyed blue araldite and coral transmit light, when transmitted light is used, it is not always possible to tell whether araldite is overlain by coral or vice versa. This is not a problem when reflected light is used as surface features are viewed only. Point counting was done at a magnification of x100 (field of view 1 mm) which enabled all pore spaces to be seen as they vary in size from 100 μm to 300 μm. As skeletal elements vary in size from 50 μm to 300 μm, readings were taken approximately every 350 μm to ensure unbiased coverage. In order to determine the errors associated with the estimates of porosity, a number of thin sections were point counted eight times each.

Table A2.6 Precision of porosity measurements.

PB-2-90 BB (5)

Repetition	PS	SK	Total	PS%
1	111	62	173	64.16
2	106	67	173	61.27
3	100	75	175	57.14
4	106	69	175	60.57
5	101	74	175	57.71
6	109	66	175	62.29
7	107	68	175	61.14
8	104	70	174	59.77
Mean				60.51
STDEV				2.30
% error				**3.81**

SB-1A-88 BB (3)

Repetition	PS	SK	Tot	PS%
1	191	149	340	56.18
2	202	138	340	59.41
3	194	142	336	57.74
4	194	146	340	57.06
5	204	136	340	60.00
6	205	131	336	61.01
7	207	141	348	59.48
8	205	133	338	60.65
Mean				58.94
STDEV				1.75
% error				**2.97**

SB-10-88 DB (8)

Repetition	PS	SK	Total	PS%
1	240	99	339	70.80
2	243	99	342	71.05
3	244	95	339	71.98
4	244	97	341	71.55
5	240	101	341	70.38
6	243	95	338	71.89
7	243	95	338	71.89
8	241	95	336	71.73
Mean				71.41
STDEV				0.59
% error				**0.83**

AQ-1-88 BB (6)

Repetition	PS	SK	Tot	PS%
1	175	113	288	60.76
2	173	112	285	60.70
3	172	115	287	59.93
4	173	115	288	60.07
5	171	118	289	59.17
6	171	119	290	58.97
7	171	119	290	58.97
8	181	107	288	62.85
Mean				60.18
STDEV				1.30
% error				**2.16**

Mean % error **2.44**

Key: PS = Pore space; SK = Skeletal material; Total = PS+SK; PS% = percentage pore space, STDEV = standard deviation, % error = standard deviation as a percentage of the mean.

Results in Table A2.6 show that the maximum error was 3.81% (standard deviation as a percentage of the mean) while the mean error was 2.44%.

The porosity of bright and dull fluorescent bands: Although the differences in porosity between bright and dull bands were not significant, results nevertheless show that bright band porosity is greater than dull band porosity for PB-2-90 and KR-10-88 while the reverse is true for AQ-1-88 and SB-1A-88 (see Table A2.7).

Table A2.7 Porosity data.

Sample	PS% (n=)	range	PS% (n=)	range	U-Test	Status
	DB		**BB**			
AQ-1-88	54.04 (12)	51.98-56.10	60.11 (10)	57.82-62.40	VS	BB>DB
SB-1A-88	55.21 (8)	53.11-57.31	57.99 (6)	55.78-60.20	NS	BB>DB
PB-2-90	61.59 (5)	59.24-63.94	59.09 (5)	56.84-61.34	NS	DB>BB
KR-10-88	60.33 (6)	58.03-62.63	55.91 (7)	53.78-58.04	NS	DB>BB
	DB Bulk density	BB Bulk density				
AQ-1-88	1.31	1.17				DB>BB
SB-1A-88	1.32	1.25				DB>BB
PB-2-90	1.13	1.2				BB>DB

Key: DB = dull bands; BB = bright bands; PS% = percent pore space; (n=) the number of thin sections point counted; range = calculated using the 3.81% error calculated for estimates of porosity; U-Test = Mann-Whitney U-Test. The relevant bulk density data from Table 2.1 (see Chapter 2) is also presented for comparison.

These results were expected considering the findings in Chapter 2, section 2.3, as skeletal porosity and bulk density are inversely related (see equation (1) in Chapter 2). For example, as bright band porosity is greater than dull band porosity in AQ-1-88, bright band bulk density would be expected to be less than dull band bulk density. As fluorescent banding is synchronous on either side of the peninsula and porosity/bulk density is not, these results therefore suggest that porosity is not the main control on fluorescent banding.

How reliable is the porosity and bulk density data?: So far I have shown that the errors associated with bulk density and porosity measurements are very small. However, this does not say much about their accuracy (i.e. how close they are to the real values of bulk density and porosity). Thus, the bulk density obtained by direct measurement (see Chapter 2, Table 2.1) was compared with the bulk density calculated from porosity using equation (1) in Chapter 2.

Table A2.8 Comparing bulk density obtained by direct measurement with bulk density calculated using porosity data.

Sample	(A)	(B)	% difference between (A) and (B)
AQ-1-88 DB	1.23	1.35	8.89
AQ-1-88 BB	1.15	1.19	3.36
SB-1A-88 DB	1.17	1.32	11.36
SB-1A-88 BB	1.14	1.24	8.06
PB-2-90 DB	0.97	1.13	14.16
PB-2-90 BB	1.00	1.20	16.61
Mean			**10.42**

Key: DB = dull bands and BB = bright bands; (A) The mean bulk density determined by direct measurement (see Table 2.1); (B) = The mean bulk density calculated from porosity.

It can be seen that on average, bulk density measurements determined from porosity data are 10.42% greater than those determined by direct measurement. Although the reason for this was not absolutely determined, it could be due to 'flaking'. When the thin sections were made, the coral was first impregnated with araldite before being sectioned (thus reducing 'flaking'), and then the outer surface (were the effects of 'flaking' would have been greater), was removed during the polishing process. Thus, although thin sections were made from the same blocks that direct bulk density measurements were obtained, the effects of 'flaking' would have been greatly reduced.

Porosity and bulk density values can also be compared by calculating the density of coral aragonite (CA) using the following formula:

$$CA = \frac{Z}{Q}$$

Where Z = (100 x the mass of the coral block given in Chapter 2, Table 2.1)/ the percent skeletal material determined from data in Table A2.7; and Q = the volume of the block given in Chapter 2, Table 2.1. This formula attempts to calculate what the density of a block of coral with a fixed volume (including pore spaces) would be if it did not have any pore spaces, i.e. was solid coral aragonite.

Sample	(n=)	CA
AQ-1-88 DB	14	2.67
AQ-1-88 BB	9	2.89
SB-1A-88 DB	9	2.62
SB-1A-88 BB	12	2.71
PB-2-90 DB	11	2.52
PB-2-90 BB	8	2.43
Mean		2.64
STDEV		0.16
% error		6.04

Table A2.9 The calculated density of coral aragonite: (i.e. aragonite plus all microscopic inclusions such as organic matter and microporosity).Key: (n=) = the number of samples, CA = the estimated density of coral aragonite calculated from porosity and bulk density measurements, % error = percentage error, STDEV = standard deviation, % error = percentage error (calculated as STDEV as a percentage of the mean).

Results in Table A2.9 show that the calculated density of coral aragonite is lower than the density of pure aragonite, which is 2.94. This could be due to one or a combination of the following:

(a) The effect of microboring organisms. While the volume of a block of coral is unlikely to change significantly as a consequence of micro-boring activity, the mass of the block will decrease therefore reducing bulk density and CA in the same way as 'flaking'. However, work in Chapter 3, section 3.4, suggests that microboring activity is responsible for the removal of 1-2% $CaCO_3$ which would not be large enough to account for the differences seen in Tables A2.8 and A2.9.

(b) Constantz (1986) has shown that microporosity can also develop as a consequence of crystal growth.

(c) The presence of organic molecules would also tend to reduce the estimates of CA as organic matter is generally less dense than aragonite.

(d) Although flaking is unlikely to be the cause of this trend, it could contribute in a small way.

There were a number of individual samples in which the estimate of CA was greater than 2.94 (see AQ-1-88 BB1a, 1b, 1d, 3b; DB1c, 1e; and SB-1A-88 BB1a in Table A2.10.

Table A2.8

SB-1A-88 % SK = 44.79 % SK = 42.01

DB	Volume (cm3)	Mass (g)	Bulk density	CA	BB	Volume (cm3)	Mass (g)	Bulk density	CA
DB2 (a)	0.11	0.12	1.17	2.62	BB1 (a)	0.17	0.21	1.25	2.98
DB2 (b)	0.19	0.24	1.24	2.78	BB1 (b)	0.13	0.15	1.14	2.72
DB2 (c)	0.18	0.20	1.15	2.57	BB1 (c)	0.13	0.14	1.07	2.55
DB2 (d)	0.15	0.19	1.23	2.76	BB1 (d)	0.16	0.19	1.23	2.92
DB3 (a)	0.17	0.20	1.16	2.58	BB2 (a)	0.11	0.13	1.13	2.69
DB3 (b)	0.32	0.36	1.13	2.53	BB2 (c)	0.14	0.15	1.11	2.65
DB3 (c)	0.20	0.24	1.19	2.65	BB2 (d)	0.19	0.21	1.12	2.66
DB3 (d)	0.26	0.31	1.18	2.64	BB3 (a)	0.21	0.25	1.21	2.88
DB3 (e)	0.22	0.25	1.10	2.46	BB3 (b)	0.16	0.16	1.02	2.43
Mean				**2.62**	BB3 (c)	0.21	0.24	1.16	2.75
STDEV				0.10	BB3 (d)	0.16	0.17	1.09	2.60
% error				3.91	BB3 (e)	0.24	0.27	1.13	2.69
					Mean				**2.71**
					STDEV				0.16
					% error				5.77

PB-2-90 % SK = 38.41 % SK = 40.91

DB	Volume (cm3)	Mass (g)	Bulk density	CA	BB	Volume (cm3)	Mass (g)	Bulk density	CA
DB1 (a)	0.48	0.47	0.96	2.51	BB1 (a)	0.47	0.50	1.06	2.58
DB1 (b)	0.32	0.27	0.85	2.21	BB1 (b)	0.74	0.67	0.91	2.21
DB1 (c)	1.20	1.17	0.98	2.54	BB1 (c)	0.69	0.66	0.96	2.34
DB1 (d)	0.49	0.48	0.98	2.55	BB1 (d)	0.56	0.55	0.99	2.42
DB2 (a)	0.63	0.54	0.85	2.22	BB1 (e)	0.27	0.27	1.00	2.45
DB2 (b)	0.48	0.48	1.01	2.62	BB2 (a)	0.66	0.66	1.00	2.45
DB2 (c)	0.92	0.84	0.91	2.37	BB2 (b)	0.27	0.26	0.94	2.30
DB2 (d)	0.32	0.33	1.01	2.63	BB2 (c)	0.54	0.60	1.11	2.70
DB3 (a)	0.98	0.99	1.01	2.64	Mean				**2.43**
DB3 (b)	0.65	0.67	1.03	2.69	STDEV				0.16
DB3 (c)	0.66	0.69	1.04	2.71	% error				6.42
Mean				**2.52**					
STDEV				0.18					
% error				7.01					

Table A2.8 cont.

AQ-1-88		% SK = 45.96					% SK = 39.89		
DB	Volume cm3	Mass (g)	Bulk density	CA	**BB**	Volume cm3	Mass (g)	Bulk density	CA
DB1 (a)	0.22	0.30	1.34	2.92	BB1 (a)	0.27	0.36	1.35	3.37
DB1 (c)	0.23	0.32	1.40	3.05	BB1 (b)	0.29	0.36	1.25	3.13
DB1 (d)	0.24	0.31	1.32	2.88	BB1 (d)	0.20	0.25	1.26	3.16
DB1 (e)	0.19	0.27	1.44	3.13	BB2 (b)	0.25	0.27	1.07	2.69
DB2 (b)	0.22	0.27	1.25	2.73	BB2 (c)	0.24	0.27	1.13	2.84
DB2 (c)	0.20	0.27	1.33	2.90	BB3 (b)	0.52	0.62	1.20	3.02
DB2 (e)	0.26	0.35	1.32	2.88	BB3 (c)	0.28	0.29	1.02	2.56
DB3 (b)	0.25	0.31	1.24	2.69	BB3 (d)	0.39	0.42	1.08	2.71
DB3 (d)	0.37	0.40	1.09	2.36	BB3 (e)	0.37	0.38	1.02	2.55
DB4 (a)	0.25	0.26	1.04	2.26	Mean				**2.89**
DB4 (b)	0.26	0.28	1.06	2.31	STDEV				0.29
DB4 (c)	0.32	0.33	1.05	2.27	% error				10.12
DB5 (a)	0.35	0.37	1.06	2.30					
DB5 (b)	0.49	0.61	1.25	2.73					
Mean				**2.67**					
STDEV				0.31					
% error				11.60					

Key: % SK = percent skeletal matter determined in Table 2.3; CA = the density of coral aragonite (including organics impurities and microporosity), STDEV = standard deviation, % error = STDEV as a percentage of mean. Mass and bulk density values were taken from Chapter 2, Table 2.1. Note, the number associated with a particular sample indicates is relationship to the living coral surface (i.e. DB1 is closer to the living coral surface than DB5.

Higher than expected aragonitic densities could be produced as a result of the following:

(a) Underestimating the volume of the block. This could happen if rhombohedrons (defined as a six sided prism whose sides are parallelograms) were cut instead of cubes/cuboids.

(b) Coral dust being left behind in the blocks after jet washing.

(c) The failure of the cleaning process to remove all non-bound surface organics. Evidence provided by Gaffey and Bronnimann (1993) suggests that this could occur even after soaking in concentrated NaOCl for 24 hours. The living coral surface is the area most likely to be affected by this and it is where six of the seven samples that had a CA over 2.94 came from.

Thus, the CA values obtained using equation (2) appear to be a playoff between the processes that reduce bulk density and the processes that enhance it. The fact that most samples have CA values below that of aragonite strongly suggested that flaking, the presence of organics in the skeleton and microporosity (both natural and due to microboring activity) outweighed the processes that could result in CA values being greater than 2.94.

APPENDIX 3. MICROPOROSITY

A3.1 The effect that increasing the number of readings in a fixed area from 289 to 1225 has on estimates of microporosity: In order to determine whether increasing the number of counts taken from 289 to 1225 made a significant different to the estimate of microboring activity in the coral skeleton, 10 of the 20 backscatter images available for each sample were chosen at random and point counted. A Mann-Whitney U-test was then performed for individual samples at 289 and 1225.

Table A3.1 Determining the best method of estimating microboring activity in TS-10-88.

BB5	PS	SK	Borings	Total	B % SK	**BB5**	PS	SK	Borings	Total	B % SK
1	85	200	4	289	2.00	1	360	843	22	1225	2.61
3	130	157	2	289	1.27	3	541	672	12	1225	1.79
5	186	103	0	289	0.00	5	807	415	3	1225	0.72
7	128	160	1	289	0.63	7	477	741	7	1225	0.94
8	196	91	2	289	2.20	8	845	371	9	1225	2.43
10	154	130	5	289	3.85	10	652	546	27	1225	4.95
12	213	76	0	289	0.00	12	922	298	5	1225	1.68
14	195	94	0	289	0.00	14	829	392	4	1225	1.02
16	228	60	1	289	1.67	16	985	232	8	1225	3.45
17	182	104	3	289	2.88	17	782	430	13	1225	3.02
Mean					**1.45**						**2.26**

Mann-Whitney U-Test

B % SK	Rank	B % SK	Rank
2.61	15	2.00	12
1.79	11	1.27	8
0.72	5	0.00	1
0.94	6	0.63	4
2.43	14	2.20	13
4.95	20	3.85	19
1.68	10	0.00	1
1.02	7	0.00	1
3.45	18	1.67	9
3.02	17	2.88	16
Sum	**123**	Sum	**84**

Critical values for a two tail test are given below:

U1	32
U2	71
99%	16
95%	23
90%	27

Table A3.1 cont.

BB7	PS	SK	Borings	Total	B % SK	BB7	PS	SK	Borings	Total	B % SK
BB7 2	224	63	2	289	3.17	2	968	253	4	1225	1.58
BB7 3	180	107	2	289	1.87	3	766	455	4	1225	0.88
BB7 4	168	120	1	289	0.83	4	724	496	5	1225	1.01
BB7 6	175	114	0	289	0.00	6	758	460	7	1225	1.52
BB7 7	199	88	2	289	2.27	7	836	380	9	1225	2.37
BB7 8	130	154	5	289	3.25	8	529	681	15	1225	2.20
BB7 12	73	215	1	289	0.47	12	290	929	6	1225	0.65
BB7 14	199	90	0	289	0.00	14	845	369	11	1225	2.98
BB7 17	159	127	3	289	2.36	17	665	551	9	1225	1.63
BB7 20	222	66	1	289	1.52	20	963	258	4	1225	1.55
Mean					**1.57**						**1.64**

Mann-Whitney U-Test

B % SK	Rank	B % SK	Rank
3.17	19	1.58	11
1.87	13	0.88	6
0.83	5	1.01	7
0.00	1	1.52	8
2.27	15	2.37	17
3.25	20	2.20	14
0.47	3	0.65	4
0.00	1	2.98	18
2.36	16	1.63	12
1.52	8	1.55	10
Sum	**101**	Sum	**107**

Critical values for a two tail test are given below:

U1	54
U2	48
99%	16
95%	23
90%	27

DB2	PS	SK	Borings	Total	B % SK	DB2	PS	SK	Borings	Total	B % SK
1	152	134	3	289	2.24	1	925	291	9	1225	3.09
4	221	68	0	289	0.00	4	644	566	15	1225	2.65
5	231	58	0	289	0.00	5	946	278	1	1225	0.36
8	211	75	3	289	4.00	8	884	334	7	1225	2.10
10	183	104	2	289	1.92	10	788	431	6	1225	1.39
11	172	115	2	289	1.74	11	740	475	10	1225	2.11
12	182	105	2	289	1.90	12	793	428	4	1225	0.93
15	148	139	2	289	1.44	15	632	584	9	1225	1.54
17	215	74	0	289	0.00	17	858	362	5	1225	1.38
18	202	85	2	289	2.35	18	854	366	5	1225	1.37
Mean					**1.56**						**1.69**

Table A3.1 cont.

Mann-Whitney U-Test

B % SK	Rank	B % SK	Rank
2.24	16	3.09	19
0.00	1	2.65	18
0.00	1	0.36	4
4.00	20	2.10	14
1.92	13	1.39	8
1.74	11	2.11	15
1.90	12	0.93	5
1.44	9	1.54	10
0.00	1	1.38	7
2.35	17	1.37	6
Sum	**101**	Sum	**106**

Critical values for a two tail test are given below:

U1	54
U2	49
99%	16
95%	23
90%	27

DB6	PS	SK	Borings	Total	B % SK	**DB6**	PS	SK	Borings	Total	B % SK
3	228	58	3	289	5.17	3	967	250	8	1225	3.20
4	205	81	3	289	3.70	4	888	330	7	1225	2.12
5	160	123	6	289	4.88	6	691	516	18	1225	3.49
7	163	124	2	289	1.61	7	699	519	7	1225	1.35
10	137	151	1	289	0.66	10	582	640	3	1225	0.47
12	178	109	2	289	1.83	12	739	477	9	1225	1.89
14	149	137	3	289	2.19	14	615	599	11	1225	1.84
16	71	216	2	289	0.93	16	320	896	9	1225	1.00
17	213	75	1	289	1.33	17	885	336	4	1225	1.19
19	212	76	1	289	1.32	19	884	338	3	1225	0.89
Mean					**2.36**						**1.74**

Mann-Whitney U-Test

B % SK	Rank	B % SK	Rank
5.17	20	3.20	16
3.70	18	2.12	14
4.88	19	3.49	17
1.61	10	1.35	9
0.66	2	0.47	1
1.83	11	1.89	13
2.19	15	1.84	12
0.93	4	1.00	5
1.33	8	1.19	6
1.32	7	0.89	3
Sum	**114**	Sum	**96**

Critical values for a two tail test are given below:

U1	41
U2	59
99%	16
95%	23
90%	27

Table A3.1 cont.

Summary Table

	289	**1225**
BB5	1.45	2.26
BB7	1.57	1.64
DB2	1.56	1.69
DB6	2.36	1.74
Mean	**1.74**	**1.83**

Key: PS = pore space; SK = skeletal material; B = borings greater than 4 μm, Total = (PS+SK+B); B % SK = borings as a percentage of skeletal material.

Results in Table A3.1 show that there is no significant difference in the estimates of microboring activity when 1225 counts are made instead of 289.

A3.2 The errors associated with the estimation of microboring activity: This was achieved by point counting each backscatter image eight times.

Table A3.2 Errors associated with the estimation of microboring activity (TS-8-88).

Back scatter image	Repetition (A)	Repetition (B)	Repetition (C)	Repetition (D)	Repetition (E)	Repetition (F)	Repetition (G)	Repetition (H)
1	2	3	3	2	2	3	3	3
2	3	3	4	3	3	2	3	2
3	4	4	4	4	4	4	4	4
4	2	1	0	0	2	1	1	1
5	1	1	1	1	1	1	1	1
6	0	1	0	0	0	0	0	0
7	2	2	2	2	3	2	1	1
8	0	1	1	1	1	1	1	1
9	1	1	1	1	1	1	1	1
10	5	4	4	4	4	4	4	3
11	2	2	1	2	2	2	2	1
12	2	1	2	2	2	1	2	2
13	2	4	4	5	5	4	4	4
14	2	3	3	3	2	4	3	3
15	4	5	5	4	4	4	5	4
16	1	1	1	2	2	1	1	1
17	1	2	1	1	1	2	2	2
18	1	1	1	2	2	2	3	3
19	1	0	1	0	0	0	0	0
20	2	3	2	2	3	2	2	3
Sum	38	43	41	41	44	41	43	40

Mean	41.38
STDEV	1.92
% error	4.65

Key: STDEV = standard deviation, % error = STDEV as a percentage of the mean.

Results in Table A3.2 show that the error associated with the estimation of microboring activity is 4.65%.

APPENDIX 4: THE FLUORESCENCE OF CORAL SOLUTIONS

A4.1 Should coral skeleton be crushed by hand with a pestle and mortar or can a tungsten tema be used?: Why crush coral skeleton? Coral skeleton is crushed so that equal mass samples can be easily obtained. In order to ascertain the best method of crushing coral skeleton, coral that wascrushed in a pestle and mortar by hand was compared with coral crushed in a tungsten tema. Approximately 25 g of coral from a common source was crudely crushed using a pestle and mortar so that the gains were less than 1 mm in diameter. This was done for two reasons: (1) to enable the coral to fit inside the tungsten tema, and (2) to homogenise the sample to some degree. 5 g of this material was crushed by hand using a pestle and mortar to less than 100 μm while the remaining 20 g was ground in a tungsten tema for eight minutes[1]. 2 g of coral crushed by the two methods was then dissolved with 40 mls of 1N HCl, concentrated to 20 mls by evaporation in an oven at 50°C, and then adjusted to pH 9.

[1] 20 g is the minimum amount of material that should be in the tungsten tema used in this experiment before operation.

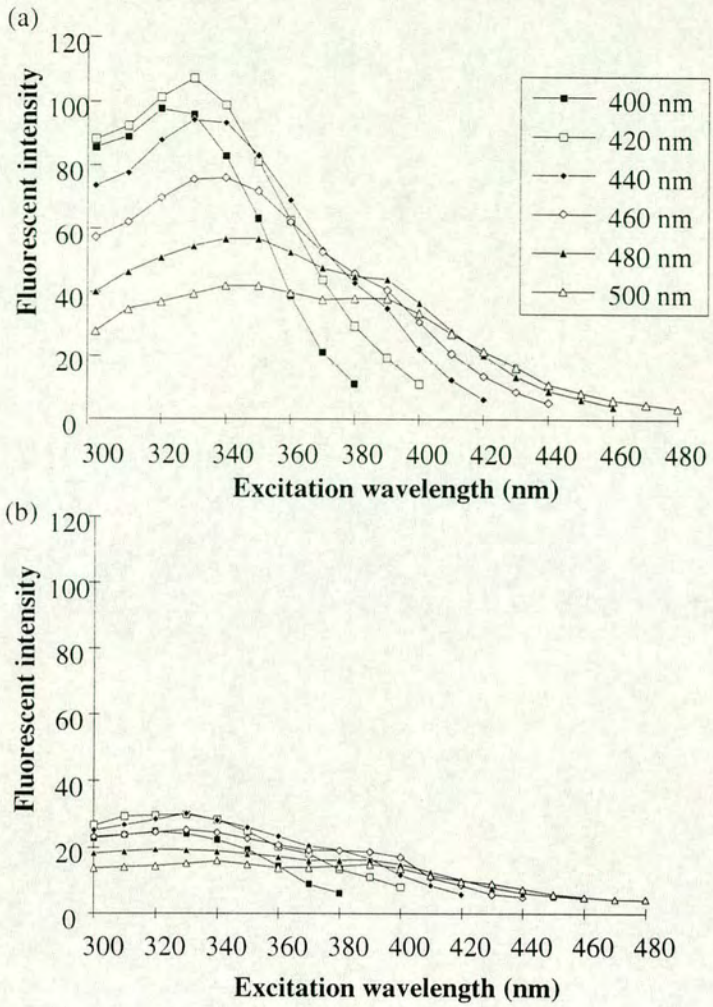

Figure A4.1 EXEM spectra of coral crushed (a) by hand in a pestle and mortar, and (b) in a tungsten tema for eight minutes.

Results in Figure A4.1 show that the fluorescent intensity of the coral solution prepared using the tungsten tema is considerably lower than the hand crushed sample. For this reason, all coral was crushed in a pestle and mortar by hand.

Appendix A4.2 Calculating the maximum mass of coral skeleton that can be dissolved with HCl and evaporated down to 20 mls without the formation of $CaCl_2$:

$CaCO_3(s) + 2HCl(aq) > Ca^{2+}(aq) + 2Cl^-(aq) + C0_2(g) + H_20(aq)$

When $CaCO_3$ is dissolved using HCl, water and $C0_2$ form as shown above. In addition, the solution also contains Ca^{2+} and Cl^- ions which will combine to form $CaCl_2$ if the solubility product of $CaCl_2$ is exceeded. The solubility of $CaCl_2$ in cold water is 745 g/1000 mls. 745g of $CaCl_2$ is the equivalent of 6.7 moles of $CaCl_2$. Thus $CaCl_2$ will form if a coral solution contains more than 6.7 moles of Ca^{2+} ions and more than 13.4 moles of Cl^- ions in 1000 mls of water. 6.7 moles of $CaCl_2$ represents a 6.7 molar calcium solution. This could be produced if 6.7 moles of $CaCO_3$ were dissolved in 1000 mls of water. 6.7 moles of $CaCO_3$ weighs 670 g. 670 g of $CaCO_3$ in 1000 mls of water is the same as 13.4 g of $CaCO_3$ in 20 mls of water. Therefore, 13.4 g is maximum amount of coral that can be dissolved and evaporated to 20 mls without the formation of $CaCl_2$ crystals. In other words, a 6.7M coral solution is the most concentrated Ca^{2+} solution that can be produced this way. If a more concentrated solution was required (i.e. to increase the concentration of fluorophores), $Ca^{2+}$and Cl^- ions would have to be removed.

A4.3 The use of buffers to control the pH of coral and industrial humic acid solutions: Adjusting the pH of coral solutions and industrially prepared humic acid solutions using acid/alkali is a slow and difficult process as the addition of even one drop of acid/alkali can result in large changes in pH. Thus, the use of buffers to adjust the pH of coral and industrially prepared humic acid solutions was considered. This work was carried out in conjunction with Dale Harris from the chemistry department, Edinburgh University. Two buffers were tested, a pH 7 sodium hydrogenphosphate buffer and a pH 9.4 borate buffer. As EXEM spectra showed that these buffers were not fluorescent and absorbed very little over the range 300 nm to 650 nm, they should not inhibit coral fluorescence unless they interact in some way with the fluorophores.

Buffer control of Aldrich humic acid: Two sets of experiments were carried out using a 17 ppm Aldrich humic acid solution. In one set, varying amounts of buffer were added to 10 mls of Aldrich humic acid solution, and in the other set, varying amounts of buffer were added to 20 mls of Aldrich humic acid solution. The Aldrich humic acid solution had a pH of 8.4 before the addition of the buffer.

Table A4.1 The pH of buffer adjusted Aldrich humic acid solutions.

Buffer	mls buffer added	pH	
		(A)	(B)
pH 7.0	5	**7.04**	7.10
	10	**7.03**	7.07
	15	**7.03**	7.06
	20	**7.01**	7.05
	30	**7.00**	7.02
pH 9.4	5	**9.19**	9.20
	10	**9.19**	9.21
	15	**9.20**	9.24
	20	**9.23**	9.29
	30	**9.30**	9.36

Key : (A) The affect buffers have when added to 10 mls of 17 ppm Aldrich humic acid solution; (B) The affect buffers have when added to 20 mls of 17 ppm Aldrich humic acid solution.

Results in Table A4.1 show that pH control (+/- 0.2) was achieved in all cases after the addition of 10 mls of buffer, although greater amounts of buffer were required to control the pH of the 20 ml Aldrich humic acid solutions. Buffer adjusted EXEM spectra were then compared to 17 ppm Aldrich humic acid solutions whose pH was adjusted with acid/alkali.

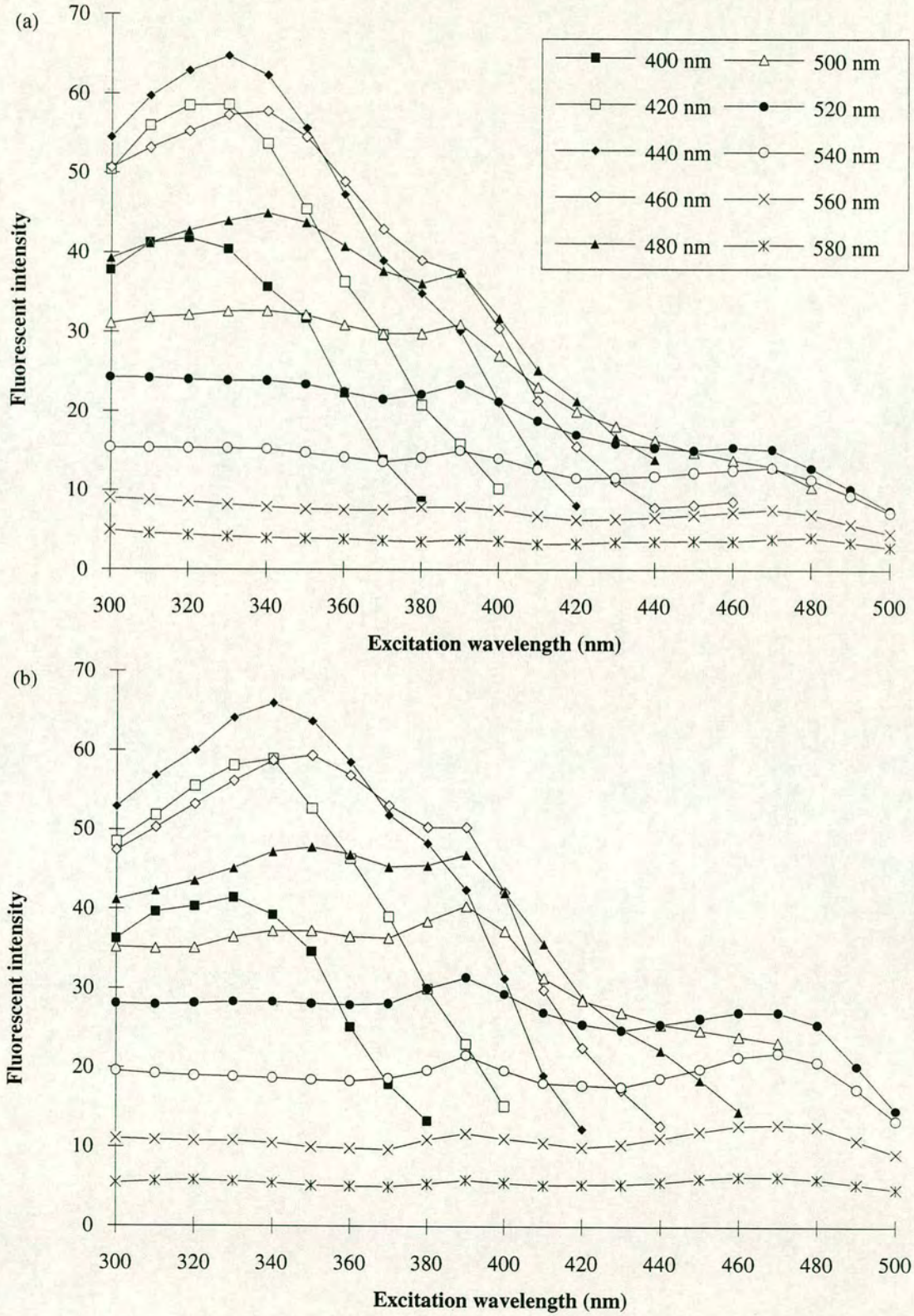

Figure A4.2 (a) a 17 ppm Aldrich Humic acid solution adjusted to pH 9 using acid/alkali, (b) a 17 ppm Aldrich humic acid adjusted to pH 9 using the borate buffer.

As shown in Figure A4.2, the EXEM spectra of Aldrich humic acid solutions adjusted to ~pH 9 with borate buffer and acid/alkali are not identical. It can be seen that the humic acid solution that was adjusted with borate buffer has a more pronounced 470 nm excitation peak than the acid/alkali adjusted solution. The 390 nm excitation peak is also enhanced in the borate buffer adjusted solution but not to the extent of the 470 nm excitation peak. This could, therefore, suggest that the borate buffer reacts/interacts with Aldrich humic acid in which case it should not be used.

Buffer control of coral solutions: After dissolution with 1M HCl, coral solutions were concentrated to 20 mls by evaporation in an oven at 50°C. At this point, the solutions generally had a pH of less than 1. The effect of adding borate buffer (pH 9.4) to coral solutions is shown in Table A4.2.

Table A4.2 The effect of adding the borate buffer to coral solutions.

mls of borate buffer added	pH
20	1.08
40	1.56
50	1.87
70	2.50
90	3.72
100	5.07
120	5.34
130	5.41
150	5.42
200	5.48

Even after the addition of 200 mls of borate buffer, pH control was not obtained. This was thought to be due to unused H^+ ions (from HCl) swamping the buffer equilibrium. By the time pH control was attained, so much buffer had been added (>200 mls) that the fluorescence was generally too weak to be detected. When the sodium hydrogenphosphate buffer (pH 7.0) was added to coral solutions, a white precipitate was formed and the pH decreased (i.e. became more acidic). The addition of more buffer resulted in continued precipitation and more acidic pH's. The precipitate was dried and identified by x-ray diffraction (XRD) as calcium hydrogenphosphate $[Ca(HPO_4)]\ .2H_2O$. The buffer equilibrium can be represented as follows:

$$H_2PO_4^- \ <----------> \ HPO_4^{2-} + H^+$$

It would appear that calcium ions react with the HPO_4^{2-} ions (from the buffer) to form $Ca(HPO_4)$ thus pushing the equilibrium reaction to the right. This process results in the generation of more HPO_4^{2-} and H^+ ions, which is why the pH became more acidic. The reaction continues until either Ca^{2+} or HPO_4^{2-} are used up. A precipitate was formed because of the low solubility of $[Ca(HPO_4)]$ $.2H_2O$ (0.316 g/litre of water). It may be possible to use the pH 7 buffer if the Ca^{2+} ions were removed. Although this could be achieved by passing the coral solutions over XAD-2 resin, results in Chapter 4, section 4.7 did not recommend the use of such resins when studying coral fluorescence.

Can the limitations of the borate buffer be overcome?: Results have shown that a large amount of borate buffer (>200 mls) is required to bring about pH control in coral solutions. One possible reason for this, as mentioned earlier, could be due to an excess of H^+ ions. In order to overcome this problem, coral solutions were adjusted to approximately pH 7 using acid/alkali before the borate buffer was added.

Table A4.3 The effect of adding borate buffer to coral solutions that were adjusted to pH 7 using acid/alkali.

mls of borate buffer added	pH
0	7.06
10	7.63
20	7.99
30	8.18
40	8.27
50	8.40
60	8.44
70	8.50
100	8.53

Results in Table A4.3 show that effective pH control was not achieved even when 100 mls of borate buffer was added. Thus, using buffers does not seem to be an effective way of controlling the pH of coral solutions.

A4.4 The effect of evaporating coral solutions to dryness: In order to determine whether coral solutions should be evaporated to dryness, two 1M coral solutions made from SB-8-88 were prepared. One was evaporated down to 20 mls and the other was evaporated to dryness. 20 mls of distilled water (DH_2O) was then added to the latter to re-dissolve the fluorophores. Both solutions were adjusted to pH 9 before spectral analysis.

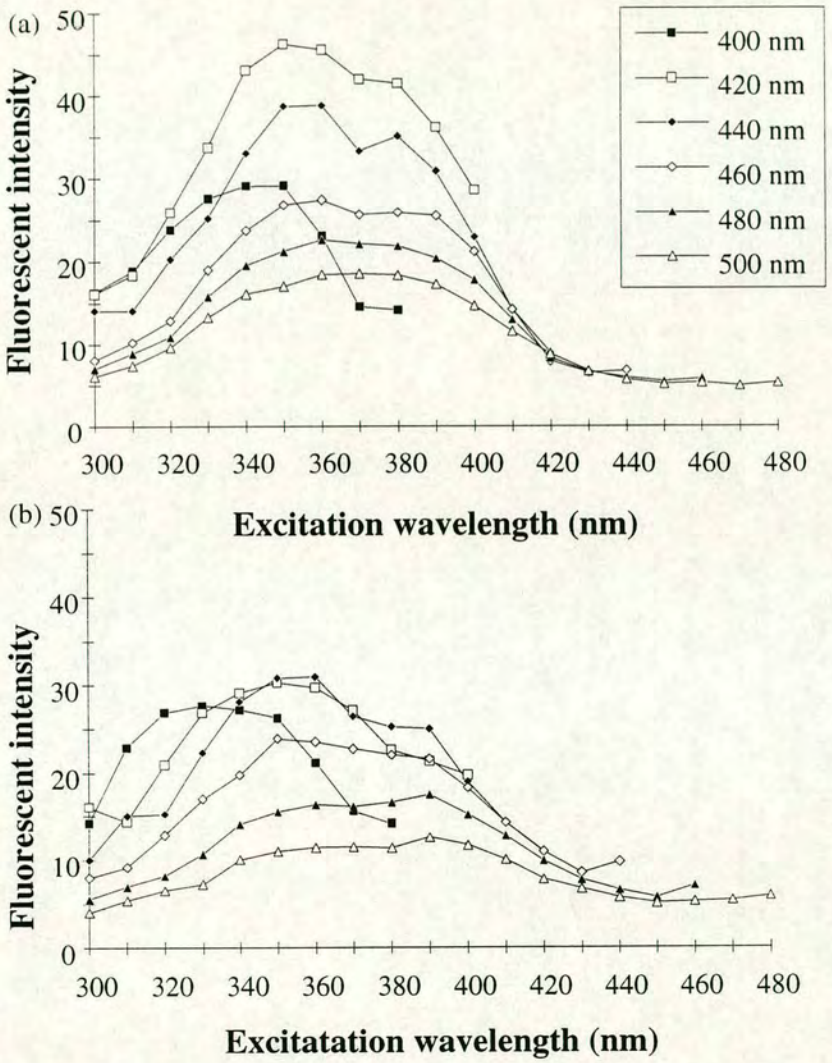

Figure A4.3 The effect of evaporating coral solutions to dryness. (a) evaporated down to 20 mls, (b) evaporated to dryness before being re-dissolved.

Results in Figure A4.3 show that the fluorescent intensity of the coral solution which was evaporated to dryness and then re-dissolved is lower than the one that was evaporated to 20 mls. This suggests that either evaporating to dryness alters the fluorescent properties of the fluorophores or that some fluorophores become

irreversibly stuck to the sides of the beaker. These results therefore suggest that coral solutions should not be evaporated to dryness.

A4.5 Scan speed errors: In order to determine the errors associated with recording EXEM spectra at different LS-5 scan speeds, a batch of six emission spectra were produced at each of the following scan speeds: 480 nm/min, 240 nm/min, 120 nm/min, 60 nm/min and 30 nm/min. For each batch of six, fresh coral solution was used from a common stock which had been adjusted to pH 9. The excitation wavelength was fixed at 320 nm and emissions were recorded from 340 nm to 500 nm. Errors were calculated at 320/400 nm as this excitation/emission wavelength combination produced the maximum variation in fluorescent intensity.

Table A4.4 Scan speed errors.

Scan speed (nm/min)	**480**	**240**	**120**	**60**	**30**
	74	77	77	76	77
	77	72	76	74	74
	77	72	75	74	77
	73	76	76	78	76
	76	77	77	76	76
	76	77	76	76	76
Mean	75.50	75.17	76.17	75.67	76.00
STDEV	1.64	2.48	0.75	1.51	1.10
% error	**2.18**	**3.30**	**0.99**	**1.99**	**1.44**

Key: STDEV = standard deviation, % error = STDEV as a percentage of the mean.

Results in Table A4.4 show that all measurements are within 3.3% of their respective means. Although 120 nm/min appears to give the best results, a scan speed of 480 nm/min was used in Chapter 4 (for coral solution work) and Chapter 6 (for solid state coral fluorescent work), as this meant that the production time for one EXEM spectrum was reduced from around an hour (at 30 nm/min) to approximately 10 minutes (at 480 nm/min) with only a small loss in precision.

A4.6 Recording long-term LS-5 spectrofluorimeter variations: Long term variations associated with the LS-5 spectrofluorimeter were calculated using the 355/400 nm water Raman scatter peak. Each figure in column (A) (see Table A4.5) represents the mean fluorescent intensity of six independent measurements made on a specified day.

Table A4.5 Long term LS-5 spectrofluorimeter variations

Date	(A)	% error
28/02/94	7.33	2.96
02/03/94	6.80	2.36
04/03/94	7.27	1.55
08/03/94	7.73	3.18
09/03/94	7.07	5.23
10/03/94	7.73	1.19
11/03/94	6.80	0.79
14/03/94	7.07	4.35
15/03/94	6.47	3.54
23/03/94	7.13	1.60
27/03/94	7.07	3.06
30/03/94	7.60	3.10
13/04/94	7.20	2.34
19/04/94	7.13	3.92
20/04/94	7.47	2.98
22/04/94	7.07	3.68
25/04/94	7.67	2.70
Mean	**7.21**	**2.85**

Key: (A) = the mean fluorescent intensity of a replicate set of six measurements made on a specified day: % error = error associated with each replicate set of six. The highlighted values represent the 2 month mean 355/400 nm water Raman scatter peak fluorescent intensity with its associated error.

A4.7 Calculating the colour of humic acid fluorescence assuming all emission wavelengths from the u/v lamp are equal over the range 330 nm to 430 nm: Using a 144 ppm Fluka humic acid solution, the colour of fluorescence was predicted assuming a polychromatic source (between 330 nm and 430 nm) whose constituent excitation wavelengths were all assumed to be of equal intensity. This was achieved by summing individually all the red, green and blue light emitted by the Fluka humic acid solution when excited over the range 330 nm to 430 nm. This then enabled the ratio of each of these to the total light emitted (i.e. red + green + blue) to be calculated thus giving the x, y and z values. These values were then compared to the x, y and z values predicted assuming that the Fluka humic acid solution was excited at 370 nm exclusively.

Table A4.6 Calculating the colour of fluorescence using LS-5 data assuming all emission wavelengths from the u/v lamp are equal over the range 330 nm to 430 nm:

(A)	X	Y	Z	
330 nm	29.54	54.20	52.29	
340 nm	34.73	63.60	60.22	
350 nm	37.90	70.81	67.16	
360 nm	40.38	77.60	71.26	
370 nm	44.73	86.86	75.19	
380 nm	52.56	103.60	85.36	
390 nm	62.68	125.59	96.20	
400 nm	66.44	134.89	96.04	
410 nm	66.33	135.29	84.19	
420 nm	69.13	140.66	74.09	
430 nm	74.05	152.09	58.60	(B)
Sum	578.46	1145.20	820.60	**2544.26**

				Exciting at 370 nm only		
	x	y	z	x	y	z
	0.23	0.45	0.32	0.22	0.42	0.36

Key: (A) = u/v lamp emission range; (B) = sum of all X, Y and Z values (note: X = sum of all red light; Y = sum of all green light; Z = sum of all blue light); x = red light as a proportion of all emissions; y = green light as a proportion of all emissions; z = blue light as a proportion of all emissions. For further details see Chapter 4, section 4.5.1).

Results in Table A4.6 show that no significant difference in the colour of fluorescence was predicted when the polychromatic source (330 nm to 430 nm) was compared with the monochromatic source (370 nm), both of which were green.

A4.8 Calculating the colour of humic acid fluorescence assuming a polychromatic source who's constituent wavelengths are not of the same intensity: This was calculated using a 144 ppm Fluka humic acid solution. PMT adjusted fluorescent intensities were multiplied by a factor which corresponded to the relative intensity of the u/v lamp at a particular excitation wavelength (see Chapter 2, Figure 2.14 for further details). This calculation, therefore, assumes that the Fluka humic acid solution is unsaturated with respect to all emission wavelengths from the u/v lamp. The x, y and z values were then calculated in the same way as the data in A4.6.

Table A4.7 Calculating the colour of fluorescence assuming a polychromatic source (u/v lamp):

Fluka humic acid values adjusted to PMT response:

	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm
330 nm	3.64	6.61	9.49	10.91	10.17	13.62	17.65	14.24	11.52	10.05
340 nm	3.63	7.40	10.36	13.13	11.86	15.75	20.91	15.97	13.87	12.15
350 nm	3.51	7.21	11.67	14.52	13.50	18.03	23.54	17.82	15.72	12.57
360 nm	3.08	6.93	11.90	15.48	14.78	20.34	26.17	19.85	17.37	12.78
370 nm	2.51	6.51	11.83	16.40	16.43	23.05	28.90	21.96	19.65	14.80
380 nm	1.81	6.02	12.54	18.74	20.03	27.67	34.31	26.65	22.76	18.16
390 nm		5.38	12.77	20.98	24.94	33.68	40.48	33.98	26.36	22.84
400 nm		3.93	11.38	20.64	27.61	36.31	41.36	36.69	29.96	24.20
410 nm			8.26	17.98	27.74	34.61	38.86	37.17	31.99	25.37
420 nm			5.61	15.01	26.41	34.94	39.12	39.05	33.98	28.26
430 nm				11.90	24.31	35.95	41.43	43.16	38.05	32.16

Relative intensity of the u/v lamp:

330 nm	340 nm	350 nm	360 nm	370 nm	380 nm	390 nm	400 nm	410 nm	420 nm	430 nm
0.01	0.03	0.48	0.91	1.00	0.93	0.77	0.39	0.11	0.01	0.01

Fluka humic acid values adjusted to u/v lamp intensity:

	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm
330 nm	1.86	3.37	4.84	5.57	5.19	6.94	9.00	7.26	5.87	5.13
340 nm	3.41	6.96	9.74	12.35	11.15	14.80	19.66	15.01	13.04	11.42
350 nm	3.51	7.21	11.67	14.52	13.50	18.03	23.54	17.82	15.72	12.57
360 nm	2.81	6.30	10.83	14.09	13.45	18.51	23.82	18.06	15.80	11.63
370 nm	1.86	4.82	8.76	12.14	12.16	17.06	21.39	16.25	14.54	10.95
380 nm	0.69	2.29	4.77	7.12	7.61	10.52	13.04	10.13	8.65	6.90
390 nm		0.59	1.41	2.31	2.74	3.71	4.45	3.74	2.90	2.51
400 nm		0.04	0.11	0.21	0.28	0.36	0.41	0.37	0.30	0.24
410 nm			0.08	0.18	0.28	0.35	0.39	0.37	0.32	0.25
420 nm			0.06	0.15	0.26	0.35	0.39	0.39	0.34	0.28
430 nm				0.12	0.24	0.36	0.41	0.43	0.38	0.32

Table A4.7 cont.

	X	Y	Z					
330 nm	15.07	27.64	26.67					
340 nm	32.64	59.79	56.61					
350 nm	37.90	70.81	67.16					
360 nm	36.75	70.62	64.85					
370 nm	33.10	64.28	55.64					
380 nm	19.97	39.37	32.44					
390 nm	6.89	13.81	10.58					
400 nm	0.66	1.35	0.96					
410 nm	0.66	1.35	0.84					
420 nm	0.69	1.41	0.74					
430 nm	0.74	1.52	0.59	(A)				
Sum	185.08	351.95	317.07	854.10				
					Exciting at 370 nm only			
	x	y	z		x	y	z	
Average	**0.22**	**0.41**	**0.37**		**0.22**	**0.42**	**0.36**	

Key: The range 330 nm to 430 nm = excitation wavelengths; the range 400 nm to 580 nm = emission wavelengths; (A) $\sum$ (X + Y + Z); x = X/(A); y = Y/(A); z = Z/(A).

Results in Table A4.7 show that even when the Fluka humic acid solution was excited with a polychromatic source of unequal excitation intensities between 330 nm and 410 nm, the predicted colour of fluorescence was not significantly different to that produced when the sample was excited at 370 nm alone (both green).

APPENDIX 6. MEASURING SOLID STATE CORAL FLUORESCENCE

A6.1 Determining the optimum angle between the u/v lamp and the photomultiplier (PMT): This was achieved by exciting a coral skeleton with 365 nm u/v light at the following angles: 30°, 40°, 50°, 60°, 70°, 80° and 90°. The emission part of the fluorescence measuring device was then moved to identify the range in which the most fluorescence was recorded.

Table A6.1 Optimum angle between u/v lamp and the PMT.

(A)	(B)	(C)
90	60	54
80	64	58
70	64	59
60	63	56
50	64	58
40	59	54
30	57	49
Sum	431	388
Mean	**61.57**	**55.43**

Key: (A) = angle between u/v lamp and coral surface; (B) largest angle between lamp and PMT giving maximum fluorescent intensity; (C) smallest angle between lamp and PMT giving maximum fluorescent intensity.

Results in Table A6.1 show that fluorescent intensity is maximised when the angle between the u/v lamp and the PMT is between 55 to 61°.

A6.2 Changing the size of the excitation strip: The size of the excitation strip was varied between 5 mm by 2 mm and 20 mm by 8 mm and its effects on coral (TS-3-93) fluorescence were recorded. The coral was excited at 365 nm and fluorescent emissions were measured at 450 nm. Different size excitation strips were produced by varying the position of the fused quartz lens while keeping the distance between the lamp and the coral surface constant.

Table A6.2 Changing the size of the excitation strip

Position along transect (cm)	(A)	(B)	(C)	(D)
0	134	265	543	1900
0.5	158	373	575	2200
1	122	306	564	2100
1.5	113	288	567	2200
2	103	285	567	2400
2.5	105	287	552	2400

Key: (A) = 5 mm by 2 mm; (B) = 10 mm by 4 mm; (C) = 15 mm by 6 mm; (D) = 20 mm by 8 mm. Fluorescent intensities in (mv).

Results in Table A6.2 show that as the size of the excitation strip increases, fluorescent intensity also increases. With the PMT amplifier on the medium sensitivity setting, background noise remained at zero for all excitation strip sizes.

A6.3 The amount of u/v light exciting the coral surface when the 254 nm and 365 nm excitation bandpass filters are used:

Using the data shown in Figure A6.1 (provided by Oriel), approximately 4000 microwatts/cm^2 (90% of the light emitted) are emitted by the mercury (argon) lamp at 254 nm, whereas approximately ~80 microwatts/cm^2 (2%) are emitted at 365 nm.

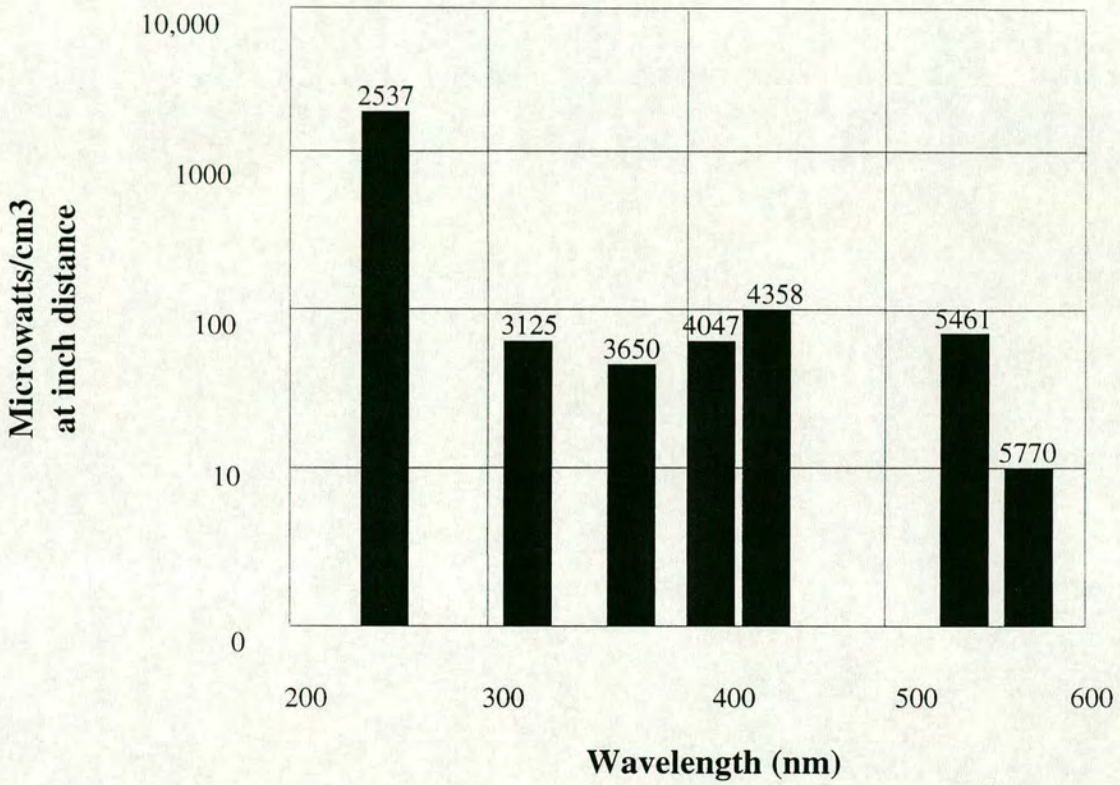

Figure A6.1 Typical irradiance of the u/v lamp used in the fluorescence measuring device. Data supplied by Oriel Corporation.

These values are reduced to ~2000 microwatts/cm^2 and ~68 microwatts/cm^2 when the effects of the short-wave filter are taken into account (see Figure A6.2).

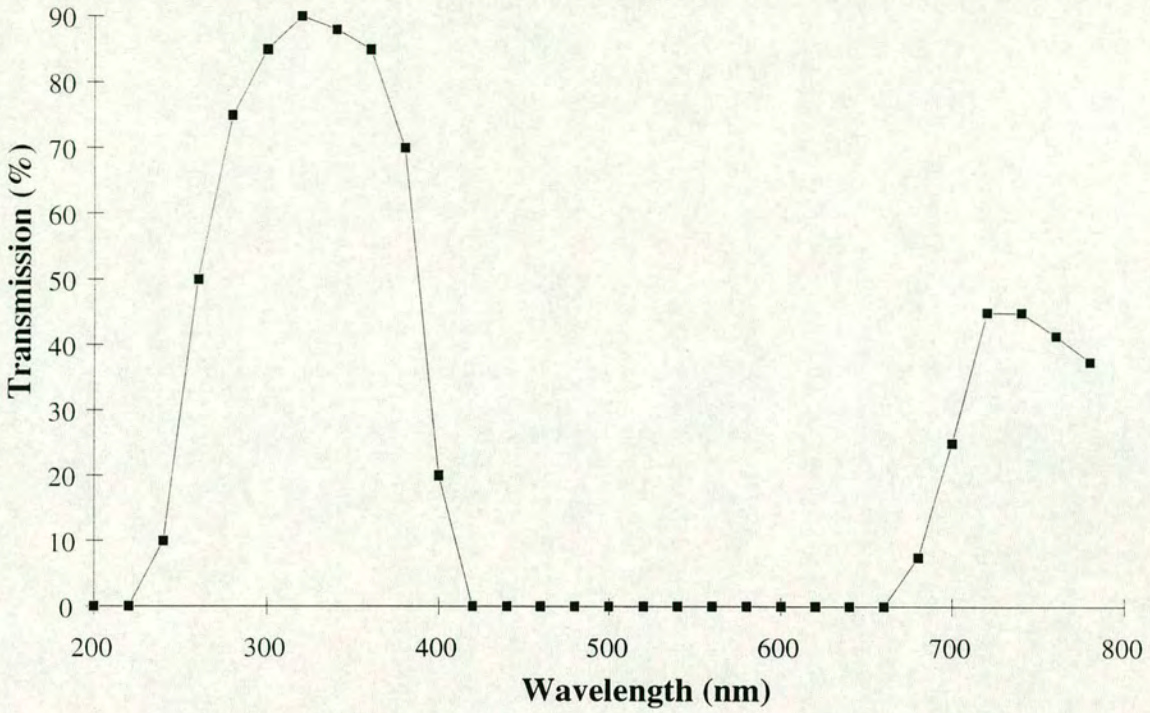

Figure A6.2 The transmission curve for the short-wave filter used in the fluorescence measuring device. Data supplied by Oriel Corporation. ~50% of 254 nm u/v light is cut out by the short wave filter and ~15% of the 365 nm u/v light.

When the effects of the excitation bandpass filters are taken into account (the 254 nm excitation bandpass filter transmits 10% of the incoming radiation between 250.5 nm and 257.5 nm, while the 365 nm excitation bandpass filter transmits 38% of the incoming radiation between 360 nm and 370 nm), the amount of 254 nm u/v light reaching the coral is reduced to ~200 microwatts/cm^2, while the amount of 365 nm u/v light is reduced to 42 microwatts/cm^2.

A6.4 Estimating the errors associated with the production of a fluorescent profile: In order to estimate the errors associated with the production of a fluorescent profile, five fluorescent profiles were obtained from one coral core (TS-3-93). Each profile started and ended in the same place. Results in Table A6.3 show that the mean error was 5.28%.

Table A6.3 Estimating the errors associated with the production of a fluorescent profile:

	RUN	1	2	3	4	5			
DLCS (cm)	Band	FLU (mv)	FLU (mv)	FLU (mv)	FLU (mv)	FLU (mv)	Mean (X)	STDEV	% error
0	D	550	710	670	660	670	652	60.17	9.23
0.2	D	620	760	770	770	760	736	65.04	8.84
0.4	D	720	890	790	770	770	788	62.61	7.95
0.6	D/B	920	1060	1060	1080	1120	1048	75.63	7.22
0.8	B	1070	1300	1320	1320	1330	1268	111.22	8.77
1	B	1050	1100	1150	1140	1190	1126	53.20	4.72
1.2	B	1150	1400	1340	1350	1380	1324	100.15	7.56
1.4	D/B	1050	1220	1250	1240	1240	1200	84.56	7.05
1.6	D	920	1050	1050	1050	1060	1026	59.41	5.79
1.8	D	940	1030	1070	1060	1060	1032	53.57	5.19
2	D	1000	1110	1140	1140	1150	1108	62.21	5.61
2.2	D	920	1010	1030	1030	1030	1004	47.75	4.76
2.4	D	780	850	860	860	860	842	34.93	4.15
2.6	D	930	1020	1050	1010	1060	1014	51.28	5.06
2.8	D	970	1040	1070	1060	1050	1038	39.62	3.82
3	B	1250	1350	1370	1360	1360	1338	49.70	3.71
3.2	B	1500	1600	1520	1620	1480	1544	62.29	4.03
3.4	D/B	1390	1500	1530	1490	1490	1480	52.92	3.58
3.6	D	1200	1270	1270	1280	1320	1268	43.24	3.41
3.8	D	1380	1450	1450	1430	1450	1432	30.33	2.12
4	D	1140	1170	1190	1190	1190	1176	21.91	1.86
4.2	D	1170	1250	1260	1220	1270	1234	40.37	3.27
4.4	B	1330	1400	1370	1400	1420	1384	35.07	2.53
4.6	B	1600	1640	1670	1680	1680	1654	34.35	2.08
4.8	B	1650	1670	1660	1710	1710	1680	28.28	1.68
5	B	1090	1470	1160	1170	1080	1194	159.47	13.36
								Mean (Y)	**5.28**

Key: DLCS = distance from living coral surface; D = dull band; B = bright band; D/B = boundary between the bands; Mean (X) = mean fluorescent intensity of the five readings taken from the same spot; STDEV = standard deviation of those five fluorescent intensity readings; Mean (Y) = the mean percentage error of all fluorescent intensities taken from the five runs.

A6.5 The colour of fluorescence predicted for Boto and Isdale's (1985) solid coral bright and dull band emission spectra: The predicted colour of fluorescent emissions from solid state bright and dull bands was calculated using Boto and Isdale's (1985) data. Two calculations were performed, one assumed that their emission data had been corrected for PMT varying response to different wavelengths, and the other assumed that their emission data had not been corrected for PMT varying response. In the calculation of the latter, the PMT correction factor used in Chapter 4 was employed. Emissions above 580 nm were not included in the calculations for two reasons: (1) a PMT correction factor was not available between 650 nm and 700 nm as the PMT could not record emissions over this range, and (2) although a PMT correction factor did exist for emission wavelengths between 580 nm and 650 nm, the low sensitivity of the PMT to emissions over this range could have introduced a large degree of error into any colour calculations.

Table A6.4 The predicted colour of Boto and Isdale's (1980) BB and DB solid state emission spectra:

Boto and Isdale's (1985) emission BB and DB spectra not PMT adjusted:

	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm
BB	46	51	76	94	95	80	62	42	33	24
DB	42	49	62	78	76	50	36	28	20	12

Adjusted to X, Y and Z factors

BB	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm	Total
X	0.00	8.67	23.56	21.62	4.75	0.00	6.82	14.28	20.13	20.64	120.47
Y	0.00	0.51	4.56	12.22	23.75	36.00	40.92	36.54	32.67	19.20	206.37
Z	2.30	18.36	132.24	147.58	76.00	28.80	8.68	1.68			415.64
										Sum	742.48
			x	y	z						
			0.16	0.28	0.56	(Blue)					

DB	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm	Total
X	0.00	8.33	19.22	17.94	3.80	0.00	3.96	9.52	12.20	10.32	85.29
Y	0.00	0.49	3.72	10.14	19.00	22.50	23.76	24.36	19.80	9.60	133.37
Z	2.10	17.64	107.88	122.46	60.80	18.00	5.04	1.12			335.04
										Sum	553.70
			x	y	z						
			0.15	0.24	0.61	(Blue)					

Table A6.4 cont.

Boto and Isdale's (1985) emission BB and DB spectra adjusted to PMT response:

	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm
BB	46	51	76	94	95	80	62	42	33	24
DB	42	49	62	78	76	50	36	28	20	12

Adjusted to PMT response

	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm
BB	46.00	52.04	80.00	106.82	125.00	123.08	110.71	100.00	110.00	120.00
DB	42.00	50.00	65.26	88.64	100.00	76.92	64.29	66.67	66.67	60.00

Adjusted to X, Y and Z factors

BB	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm	Total
X	0.00	8.85	24.80	24.57	6.25	0.00	12.18	34.00	67.10	103.20	280.94
Y	0.00	0.52	4.80	13.89	31.25	55.38	73.07	87.00	108.90	96.00	470.81
Z	2.30	18.73	139.20	167.70	100.00	44.31	15.50	4.00			491.75
										Sum	1243.5
				x	y	z					
				0.23	0.38	0.40	(Green)				

DB	400 nm	420 nm	440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm	580 nm	Total
X	0.00	8.50	20.23	20.39	5.00	0.00	7.07	22.67	40.67	51.60	176.12
Y	0.00	0.50	3.92	11.52	25.00	34.62	42.43	58.00	66.00	48.00	289.98
Z	2.10	18.00	113.56	139.16	80.00	27.69	9.00	2.67			392.18
										Sum	858.28
				x	y	z					
				0.21	0.34	0.46	(Blue/green)				

Key: 400 nm to 580 nm = emission wavelength range, Total = the sum of the individual X, Y and Z components, Sum = the total X value + the total Y value + the total Z value.

Results show that while the predicted colour of fluorescence was blue for the unadjusted bright and dull band data, a difference in the colour of fluorescence was predicted for the PMT adjusted data, with bright bands being green and dull bands being blue/green.